Table 4. Effect of OPG on the size, bone mineral content (BMC) and bone mineral density (BMD) of the tibia, femur and humerus

Bone	Drug	Length (mm)	BMC (g)	BMD (g/cm^2)
Tibia	TS-vehicle	37.3 ± 0.60	0.169 ± 0.020	0.095 ± 0.008
	TS-OPG	37.4 ± 0.82	0.184 ± 0.019[a]	0.101 ± 0.007[a]
	NL-vehicle	37.7 ± 0.59	0.185 ± 0.013	0.105 ± 0.005[b]
	NL-OPG	38.1 ± 0.52	0.189 ± 0.006	0.111 ± 0.003[bc]
Femur	TS-vehicle	33.7 ± 0.73	0.192 ± 0.025	0.116 ± 0.011
	TS-OPG	34.0 ± 0.78	0.216 ± 0.027[b]	0.123 ± 0.012
	NL-vehicle	33.6 ± 0.50	0.200 ± 0.004	0.134 ± 0.003[bc]
	NL-OPG	33.9 ± 0.29	0.218 ± 0.003	0.142 ± 0.003[bd]
Humerus	TS-vehicle	25.3 ± 0.46	0.104 ± 0.013	0.108 ± 0.010
	TS-OPG	25.4 ± 0.65	0.111 ± 0.012	0.112 ± 0.009
	NL-vehicle	25.9 ± 0.28[a]	0.101 ± 0.005	0.107 ± 0.005
	NL-OPG	26.1 ± 0.08[bc]	0.106 ± 0.005	0.117 ± 0.005

Values are mean ± SD
[a] $P < 0.05$, when compared with TS-vehicle (ANOVA Fisher's PLSD test)
[b] $P < 0.01$, when compared with TS-vehicle (ANOVA Fisher's PLSD test)
[c] $P < 0.05$, when compared with TS-OPG (ANOVA Fisher's PLSD test)
[d] $P < 0.01$, when compared with TS-OPG (ANOVA Fisher's PLSD test)

Table 5. Bone mineral content (BMC) and bone mineral density (BMD) of each region of the femur

	Drug	Region 1	Region 2	Region 3	Region 4
BMC (g)	TS-vehicle	0.054 ± 0.007	0.035 ± 0.005	0.050 ± 0.007	0.054 ± 0.009
	TS-OPG	0.062 ± 0.009[b]	0.041 ± 0.006[b]	0.057 ± 0.007[b]	0.056 ± 0.009
	NL-vehicle	0.061 ± 0.002[a]	0.037 ± 0.002	0.050 ± 0.002[c]	0.053 ± 0.005
	NL-OPG	0.065 ± 0.001[b]	0.040 ± 0.002	0.053 ± 0.002	0.060 ± 0.002
BMD (g/cm^2)	TS-vehicle	0.105 ± 0.012	0.097 ± 0.011	0.145 ± 0.016	0.121 ± 0.011
	TS-OPG	0.114 ± 0.014[a]	0.106 ± 0.010[a]	0.154 ± 0.013	0.124 ± 0.015
	NL-vehicle	0.131 ± 0.005[bd]	0.109 ± 0.006[a]	0.155 ± 0.006	0.143 ± 0.004[bd]
	NL-OPG	0.138 ± 0.004[bd]	0.115 ± 0.003[b]	0.165 ± 0.005[a]	0.152 ± 0.004[bd]

Values are mean ± SD
[a] $P < 0.05$, when compared with TS-vehicle (ANOVA Fisher's PLSD test)
[b] $P < 0.01$, when compared with TS-vehicle (ANOVA Fisher's PLSD test)
[c] $P < 0.05$, when compared with TS-OPG (ANOVA Fisher's PLSD test)
[d] $P < 0.01$, when compared with TS-OPG (ANOVA Fisher's PLSD test)

23]. Kodama et al. [10, 21] reported that agents reversing the inhibition of periosteal bone formation are required to restore normal bone strength and geometry in situations of prolonged mechanical unloading.

These findings suggest that bone-forming agents might be more useful than agents that inhibit bone resorption for prevention of bone loss or decrease in bone strength of the diaphysis induced by long-term unloading over 14 days. For example, high-dose intermittent administration of human PTH, a bone-forming agent that prevents change of trabecular bone volume, structure, and bone formation rate induced prolonged mechanical unloading (2–6 wk) in neurectomized mice [27].

Moreover, Kodama et al. demonstrated that pamidronate, an antiresorptive agent, increased the overall BMD as well as femoral metaphysis. However, it showed almost no effect on the BMD of the diaphysis or bone strength of the femoral midshaft in either control or tail-suspended rats [10]. In the present study, treatment with OPG in tail-suspension increased not only the total BMC of the tibia and femur, but also BMC and BMD of the femoral diaphysis. Moreover, OPG prevented reduction in bone strength measured by three-point bending of the femoral midshaft during tail suspension. Actually, treatment with OPG in tail suspension brought the BMD and bone strength of the midshaft to a level that was nearly equal to that in the NL-vehicle group. These findings differed from those in treatment with bisphosphonates. Inhibitory effects of bisphosphonates on bone mineralization may explain this difference. Francis et al. [24] reported that bisphosphonates inhibited formation of calcium phosphate crystals *in vitro* [24]. Apseloff et al. [25] reported that aminohydroxybutane bisphosphonate (AHBuBP) could not prevent reduction in bone

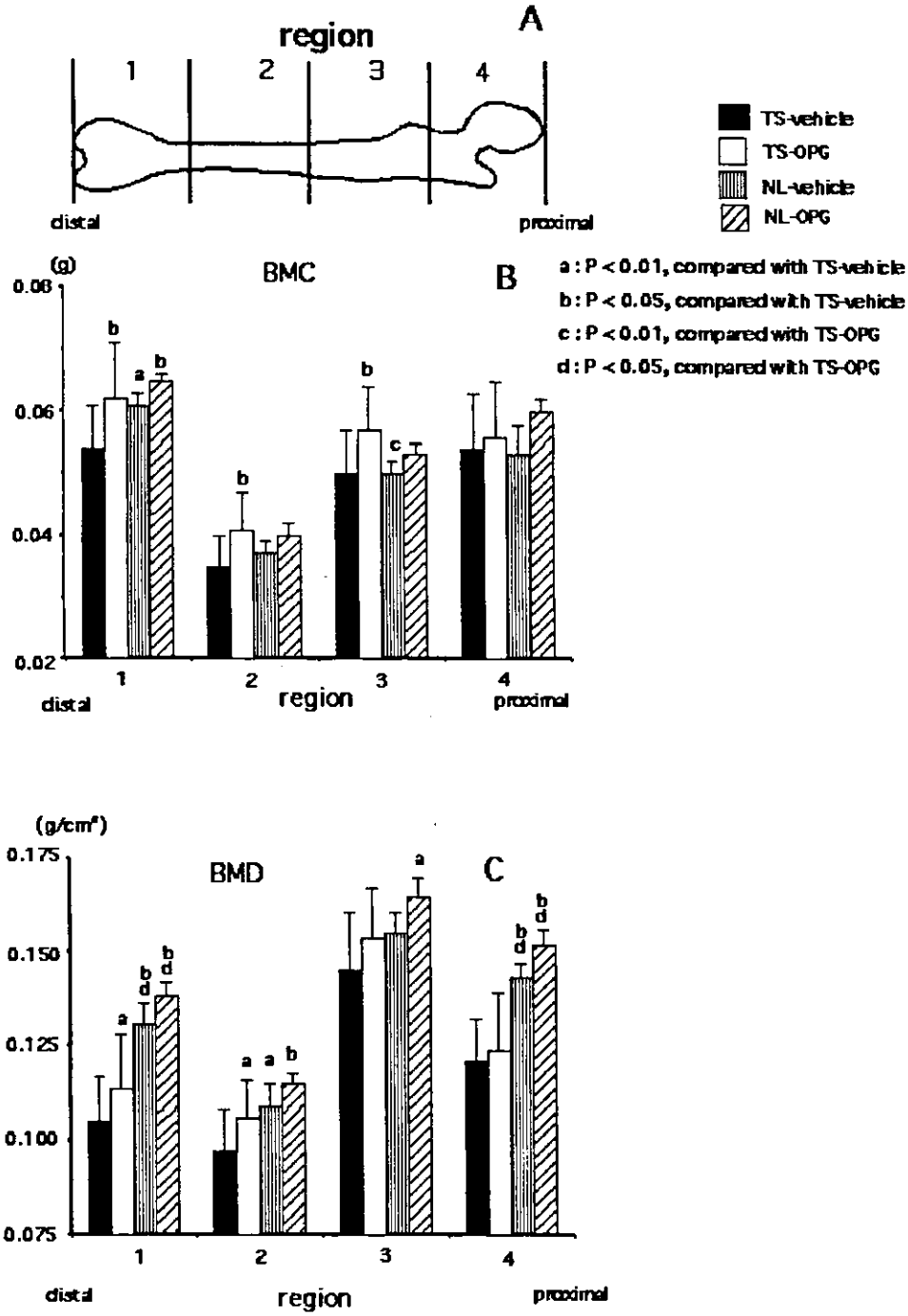

Fig. 1. Femora were divided into 4 equal length as shown (A), and the BMD (B) and BMC (C) of each region were measured by DXA. Data were reported as mean ± SD. Among the tail-suspended groups, significant difference in BMD and BMC of region 1-2 were shown. The BMDs of regions 2-3 in the TS-OPG group were nearly equal to that in the NL-vehicle group.

strength of the femoral diaphysis. They suggested that newly formed bone was weaker in AHBuBP-treated rats.

Along with bisphosphonate, OPG is a potent bone resorption inhibitor, but treatment with high doses of OPG alone increased BMD and bone volume and caused neither histopathological abnormalities in other tissues nor significant changes in the number of leukocytes, erythrocytes, reticulocytes, or platelets [11]. Therefore, OPG may act solely on osteoclasts by inhibiting their formation. This inhibition may, at least in part, explain the difference between the two potent bone resorption inhibitors.

In summary, the present findings indicate that treatment with OPG can prevent decrease in BMD not only in cancellous bone but also in cortical bone of the femur. Treatment with OPG can prevent decrease in bone strength at the diaphysis of the femur. These findings suggest that OPG may be useful to treat osteoporosis induced by skeletal unloading, including space flight,

Table 6. Mechanical strength of femur measured using the three-point bending method

Group	Ultimate force (N)
TS-vehicle	82.3 ± 11.4
TS-OPG	95.1 ± 8.0[a]
NL-vehicle	97.8 ± 13.6[a]
NL-OPG	123.0 ± 14.7[abc]

Values are mean ± SD
[a] $P < 0.01$, when compared with TS-vehicle (ANOVA Fisher's PLSD test)
[b] $P < 0.01$, when compared with TS-OPG (ANOVA Fisher's PLSD test)
[c] $P < 0.01$, when compared with NL-vehicle (ANOVA Fisher's PLSD test)

and especially to prevent bone fracture during and after space flight.

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References

1. Racin R, Gordon SL, Lymn RW, Todd PW, Frey MA, Sulzma FM (1993) Effects of space flight on the musculoskeletal system: NIH and NASA future directions. FASEB J 7:396–398
2. Bikle DD, Halloran BP, Morey-Holton ER (1997) Space flight and the skeleton: lessons for the earthbound. Endocrinologist 7:10–22
3. Vico L, Novikov VE, Very JM, Alexandre C (1991) Bone histomorphometric comparison of rat tibial metaphysis after 7-day tail suspension vs. 7-day space flight. Aviat Space Environ Med 62:26–31
4. Weinreb M, Rodan GA, Thompson DD (1989) Osteopenia in the immobilized rat hind limb is associated with increased bone resorption and decreased bone formation. Bone 10:187–194
5. Sevastikoglou JA, Mattsson S (1976) Changes in composition and metabolic activity of the skeletal parts of the extremity of the adult rat following immobilization in a plaster cast. Acta Chir Scand (Suppl) 467:21–25
6. Maeda H, Kimmel DB, Raab DM, Lane NE (1993) Musculoskeletal recovery following hindlimb immobilization in adult female rats. Bone 14:153–159
7. Halloran BP, Bikle DD, Harris J, Tanner S, Curren T, Morey-Holton E (1997) Regional responsiveness of the tibia to intermittent administration of parathyroid hormone as affected by skeletal unloading. J Bone Miner Res 12:1068–1074
8. Halloran BP, Bikle DD, Harris J, Autry CP, Currier PA, Tanner S, Patterson-Buckendahl Morey-Holton E (1995) Skeletal unloading induces selective resistance to the anabolic actions of growth hormone on bone. J Bone Miner Res 10:1168–1176
9. Machwate M, Zerath E, Holy X, Pastoureau P, Marie PJ (1994) Insulin-like growth factor-I increases trabecular bone formation and osteoblastic cell proliferation in unloaded rats. Endocrinology 134:1031–1038
10. Kodama Y, Nakayama K, Fuse H, Fukumoto S, Kawahara H, Takahashi H, Kurokawa T, Sekiguchi C Nakamura T, Matsumoto T (1997) Inhibition of bone resorption by pamidronate cannot restore normal gain in cortical bone mass and strength in tail-suspended rapidly growing rats. J Bone Miner Res 12:1058–1067
11. Yasuda H, Shima N, Nakagawa N, Mochizuki S, Yano K, Fujise N, Sato Y, Goto M, Yamaguchi K, Kuriyama M, Kanno T, Murakami A, Tsuda E, Morinaga T, Higashio K (1998) Identity of osteoclastogenesis inhibitory factor (OCIF) and osteoprotegerin (OPG): a mechanism by which OPG/OCIF inhibits osteoclastogenesis *in vitro*. Endocrinology 139:1329–1337
12. Globus RK, Bikle DD, Morey-Holton E (1984) Effects of simulated weightlessness on bone mineral metabolism. Endocrinology 114:2264–2270
13. Globus RK, Bikle DD, Morey-Holton E (1986) The temporal response of bone to unloading. Endocrinology 118:733–742
14. Fujimoto S, Kubo T, Tanaka H, Miura M, Seino Y (1995) Urinary pyridinoline and deoxypyridinoline in healthy children and in children with growth hormone deficiency. J Clin Endocrinol Metab 80:1922–1928
15. Omi (1995) The effect of ovariectomy on bone metabolism in rats. Bone 17(suppl):157S–162S
16. Morey-Holton ER, Globus RK (1998) Hindlimb unloading of growing rats: a model for predicting skeletal changes during space flight. Bone 22:83S–88S
17. Wronski TJ, Morey-Halton ER (1987) Skeletal response to simulated weightlessness: a comparison of suspension techniques. Aviat Space Environ Med 58:63–68
18. Machwate M, Zerath E, Holy X, Hott M, Godet D, Lomri A, Marie PJ (1995) Systemic administration of transforming growth factor-ß2 prevents the impaired bone formation and osteopenia induced by unloading in rats. J Clin Invest 96:1245–1253
19. Ohmori S, Kurokouchi K, Kanda K, Ito T, Kawano S, Murata Y, Seo H (1996) Changes in urinary excretion of deoxypyridinoline in tail-suspended rats: effect of bisphosphonate, YH529. Ann Res Inst Environ Med 47:3–6
20. Ito T, Ohmori S, Kanda K, Kawano S, Murata Y, Seo H (1994) Changes in the urinary excretions of markers for bone metabolism-comparison of pyridinoline and hydroxyproline. Ann Res Inst Environ Med 45:12–13
21. Kodama Y, Kurokawa T, Nakayama K, Matsumoto T (1997) Effects of skeletal unloading on bone: analysis using tail-suspended rats. J Jpn Soc Bone Morphom 7:17–22
22. Matsumoto T, Nakayama K, Kodama Y, Fuse H Nakamura T, Fukumoto S (1998) Effect of mechanical unloading and reloading on periosteal bone formation and gene expression in tail-suspended rapidly growing rats. Bone 22:89S–93S
23. Nakayama K, Kodama Y, Fukumoto S, Fuse H, Takahashi H, Kawaguchi H, Kurokawa T, Sekiguchi C, Nakamura T, Matsumoto T (1995) Mechanical unloading reduces cortical bone volume and strength by a decrease in bone formation. J Bone Miner Res 10(suppl1):S387
24. Francis MD, Russell RG, Fleisch H (1969) Disphosphonates inhibit formation of calcium phosphate crystals *in vivo* and pathological calcification *in vitro*. Science 165899:1264–1266
25. Apseloff G, Girten B, Weisbrode SE, Walke M, Stern LS, Krecic ME, Gerber N (1993) Effects of aminohydroxybutane bisphosphonate on bone growth when administered after hindlimb bone loss in tail-suspended rats. J Pharmacol Exp Ther 267:515–521
26. Bekker PJ, Holloway D, Nakanishi A, Arrighi M, Leese PT, Dunstan CR (2001) The effect of a single dose of osteoprotegerin in postmenopausal women. J Bone Miner Res 16:348–360
27. Sakai A, Sakata T, Ikeda S, Uchida S, Okazaki R, Norimura T, Hori M, Nakamura T (1999) Intermittent administration of human parathyroid hormone (1–34) prevents immobilization-related bone loss by regulating bone marrow capacity for bone cells in ddY mice. J Bone Miner Res 14:1691–1699

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Increase in Serum Levels of Autoantibodies after Attack of Seasonal Allergic Rhinitis in Patients with Graves' Disease

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Key Words
Allergic rhinitis · Graves' disease · Autoantibodies · Pollinosis · Cedar pollen

Abstract
Background: The prevalence of allergic disease is increasing worldwide, but its influence on the clinical course of autoimmune diseases is unknown. ***Objective:*** The purpose of this study was to assess the effect of seasonal allergic rhinitis on the clinical course of Graves' disease, which has been considered a Th2-dominant autoimmune disease. ***Methods:*** Ten patients with Graves' disease, who were considered to be in a state of remission or near remission, were serially examined for 18 months starting from August. Five of them had seasonal allergic rhinitis due to Japanese cedar pollen, and the remaining patients had no such allergic disorders. Peripheral eosinophil counts, serum concentrations of cedar-pollen-specific IgE, anti-TSH-receptor antibody, anti-thyroid-peroxidase antibody and antithyroglobulin antibody were assessed at 2- to 4-month intervals. Serum thyroid hormones and TSH levels were also measured to evaluate disease activity. ***Results:*** All patients with pollinosis had attacks of allergic rhinitis caused by cedar pollen in early March. Subsequently, peripheral eosinophil counts, pollen-specific IgE activity and serum levels of anti-thyroid-peroxidase and antithyroglobulin autoantibodies markedly increased. Serum levels of anti-TSH-receptor antibody increased in 3 patients in association with an increase in serum thyroid hormones but were always negative in 2 patients. The control patients without pollinosis showed no consistent change of these parameters. ***Conclusions:*** Seasonal allergic rhinitis aggravated the clinical course of Graves' disease and induced an increase in serum antithyroid autoantibody concentrations as well as an increase in pollen-specific IgE concentration. These data suggest that environmental antigens induce not only local allergic reactions, but also stimulate thyroid immune reactions toward Th2 proliferation, and finally aggravate Th2-dependent autoimmune thyroid disease.

Introduction

Allergic rhinitis is a Th2-dependent disease and may influence the clinical course of other Th2-predominant diseases. Previously we had found that seasonal allergic

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rhinitis induced by Japanese cedar pollen aggravated Graves' disease and patients frequently suffered relapses of Graves' thyrotoxicosis [1]. Moreover, some patients develop a clinical onset of Graves' disease after an attack of allergic rhinitis [2].

Graves' thyrotoxicosis is induced by thyroid-stimulating anti-TSH-receptor antibodies, and thus humoral immunity is more important than cellular immunity [3]. In patients with Graves' disease, peripheral eosinophil counts were significantly increased [4]. Furthermore, serum concentrations of interleukin (IL) 5 [5], soluble CD30 [6], IgE [7] and eosinophil-derived neurotoxin were increased [8]. Moreover, it has recently been clarified that the association of seasonal allergic rhinitis was frequent in Graves' disease and rare in painless thyroiditis [9]. These data strongly suggest that Graves' disease is a Th2-predominant disorder, although there have been contradictory reports, i.e. that the Th1-driven response is important for the initial phase of Graves' disease [10–13].

Allergic diseases are often associated with Th2-dominant autoimmune disease, such as systemic lupus erythematosus [14–16]. However, little is known about the influence of seasonal allergic rhinitis on the clinical course of autoimmune diseases and on the production of autoantibodies. It is well known that Japanese cedar pollens are heavily disseminated at the end of February to early March and frequently induce cedar pollinosis [9, 17]. In this study, we examined the effects of Japanese cedar pollinosis on autoantibody production in order to clarify the mechanism of aggravation of Graves' disease.

Methods

Patients

We examined 10 female patients with Graves' disease who were considered to be in a state of near remission under a maintenance dose of 2.5 or 5 mg of methimazole per day or in remission after antithyroid drug therapy. Of these, 5 patients had their disease complicated by seasonal allergic rhinitis due to Japanese cedar pollen. None of these patients received any medications, including immunosuppressive drugs, for allergic rhinitis. The other 5 patients had no such allergic diseases. The clinical course was examined in these patients for 18 months starting from August 1999 or August 2000. Physical examination and laboratory tests were performed every 2–4 months. Informed consent was obtained from all patients.

Measurements

Peripheral eosinophil counts were made using an automated leukocyte differential system (Total Hematology Management System NE-7000; Sysmex Co., Kobe, Japan) [4]. The serum levels of IgE specific to Japanese cedar pollen were measured with UniCAP100 (Pharmacia and Upjohn Diagnostics AB, Uppsala, Sweden) using a fluorescence enzymatic immunoassay. The normal cutoff value was 0.34 UA/ml. Anti-TSH-receptor antibody was measured with a commercial radioreceptor assay kit (Dyno test TRAb Human Kit, Yamasa Corporation, Tokyo, Japan) [18]. This assay uses human recombinant receptors, and the cutoff value was 1.0 IU/l.

Antithyroglobulin antibody and anti-thyroid-peroxidase antibody were measured with commercial radioimmunoassay kits: TgAb (Cosmic Corporation, Tokyo, Japan) and TPO Antibody Kit 'Eiken' (Eiken Chemical Co. Ltd., Tokyo, Japan), respectively. Normal cutoff values were 0.3 U/ml and 0.1 IU/ml, respectively. Activities of anti-streptolysin-O antibody were measured by nephelometry for latex agglutination using LX-3000 (Eiken Chemical Co.). The lowest detection limit was 30 IU/ml.

Serum concentrations of IL-5 and IL-13 were measured with commercial enzyme immunoassay kits, Quantakine human IL-5 and IL-13 immunoassays (R&D Systems Inc., Minneapolis, Minn., USA), respectively. The minimum detectable doses of IL-5 and IL-13 were less than 3.0 and 32 pg/ml, respectively.

In order to examine the IgE-associated anti-TSH-receptor antibodies, 300 μl sera obtained from patients 1, 4 and 5 were incubated with anti-IgE-antibody-coated magnetic particles overnight at 4°C. The mixture was centrifuged at 3,000 rpm for 15 min, and the obtained IgE-depleted sera were assayed for activities of anti-TSH-receptor antibodies, anti-thyroid-peroxidase antibodies and antithyroglobulin antibodies.

Statistical Analysis

Comparison of each parameter at different times was performed by the paired t test.

As for antithyroglobulin antibody, antibody values were transformed logarithmically, and the obtained values were compared between the two different times by the paired t test.

Results

Age, sex and clinical data of patients at various times are summarized in table 1. Patients with and without seasonal allergic rhinitis were categorized as groups A and B, respectively. Four patients had been receiving minimal maintenance doses of methimazole, and the remaining patients were followed without any medication. Even during attacks of allergic rhinitis, no patients received any antiallergic drugs. As expected, cedar-pollen-specific IgE was positive in all patients in group A and negative in group B patients. Anti-TSH-receptor antibodies were weakly positive in 2 patients in group A and in 4 patients in group B in November–January before an attack of allergic rhinitis (table 1). Anti-thyroid-peroxidase antibodies were positive in all patients except case 3. Antithyroglobulin antibodies were positive in all patients except case 1 in November–January. For each parameter, 'basal values' in November–January were compared to the values around the peak time of increase in group A. Peripheral eosinophil counts and serum levels of cedar-pollen-specific IgE were significantly increased in April–May and in

Table 1. Clinical data in patients with Graves' disease

Case	Age years	Peripheral eosinophil counts, n/mm³		Cedar-pollen-specific IgE, UA/ml		Anti-TSH-receptor antibody, IU/l		Anti-thyroid-peroxidase antibody, IU/ml		Anti-thyroglobulin antibody, U/ml		Thyroid therapy in Apr–July
		Nov–Jan	Apr–May*	Nov–Jan	May–Jun*	Nov–Jan	Jun–Oct	Nov–Jan	Sept–Oct*	Nov–Jan	Sept–Oct*	
Group A												
1	34	211	583	8.9	27.0	1.3	3.2	154	386	<0.3	0.7	none
2	19	127	358	14.5	26.4	<1.0	<1.0	83.5	965	0.7	2.7	methimazole 2.5 mg/day
3	58	234	302	26.2	37.7	<1.0	<1.0	<0.1	<0.1	11.2	137	none
4	54	143	340	12.4	16.4	1.2	52.9	250	673	51.5	176	none
5	40	153	n.t.	15.9	17.8	<1.0	7.5	25.8	455	0.3	9.7	none
		Nov–Jan	Apr–May	Nov–Jan	May–Jan	Nov–Jan	Jun–Oct	Nov–Jan	Sept–Oct	Nov–Jan	Sept–Oct	
Group B												
6	27	30	68	<0.34	<0.34	<1.0	<1.0	272	1,110	86.4	73.3	none
7	58	225	231	<0.34	<0.34	2.3	1.5	158	82.3	10.2	7.5	methimazole 2.5 mg/day
8	36	133	151	<0.34	<0.34	1.2	2.1	5,678	6,747	20.1	28.3	none
9	25	70	24	<0.34	<0.34	1.2	<1.0	273	116	12.8	5.1	methimazole 2.5 mg/day
10	28	372	283	<0.34	<0.34	1.7	2.9	1,214	1,359	7.2	6.5	methimazole 5 mg/day

Group A = Patients with Graves' disease complicated by seasonal allergic rhinitis; group B = patients without seasonal allergic rhinitis; n.t. = not tested.
* $p < 0.05$: significant difference between values at two different time points.

May–June, respectively (table 1). Activities of anti-thyroid-peroxidase antibody were also significantly increased when an antibody-negative patient (case 3) was excluded. Activities of antithyroglobulin antibody were also significantly increased when statistical analysis was performed using logarithmically transformed antibody values.

Serial changes in peripheral eosinophil counts and serum levels of pollen-specific IgE, anti-TSH-receptor antibody, antithyroglobulin antibody and anti-thyroid-peroxidase antibody in case 4 are shown in figure 1. The increase in anti-TSH-receptor antibody was remarkable, and the peak was slightly delayed compared to that of eosinophil counts and pollen-specific IgE. Increases in antithyroglobulin and anti-thyroid-peroxidase antibodies were further delayed, and their peaks were found in October. This patient had a small goiter and the increase in thyroid hormones was mild, although anti-TSH-receptor antibody increased to 60 IU/l. Since the patient rejected the readministration of methimazole, she was followed without any drugs. Thyroid function returned to normal in association with a decrease in activities of anti-TSH-receptor antibody.

Peripheral eosinophil counts increased at the time of allergic rhinitis in most patients in group A, but no consistent change was found in group B patients (fig. 2). Two patients in group A had a second slight increase in eosinophils in October. The cedar-pollen-specific IgE concentration clearly increased after attacks of seasonal allergic rhinitis in all patients (fig. 3), and a mild second increase in IgE was found around October in 3 patients in group A (fig. 3a). All patients in group B showed negative cedar-pollen-specific IgE during the observation period (fig. 3b).

Serum levels of anti-TSH-receptor antibody increased after an attack of allergic rhinitis in 3 patients in group A (fig. 4a). The remaining 2 patients showed a negative reaction during the observation period. In case 1, activities of anti-TSH-receptor antibodies increased gradually, and the serum TSH level became undetectable in association with a slight increase in serum free T_4 in September. Finally, methimazole was restarted 6 months later. In case 4, transient mild hyperthyroidism relapsed in April and May but resolved spontaneously in association with a decrease in anti-TSH-receptor antibodies. In case 5, antibody activity increased from less than 1.0 to 7.5 IU/l in June. In association with this change, thyrotoxicosis (free T_4, 2.7 ng/dl) relapsed and methimazole therapy (15 mg/day) was restarted in July. In group B, no consistent change was observed (fig. 4b).

The increase in the serum level of anti-thyroid-peroxidase antibody is shown in figure 5. All patients, except No. 3, showed a gradual increase in antibody activities in group A, although the peak was delayed from that of pollen-specific IgE or anti-TSH-receptor antibody. In group B, 2 patients (cases 6 and 8) had an increase in antibody activity in spring, but the antibody increase was observed before the attack of allergic rhinitis in case 6. The change in antithyroglobulin antibodies was more marked (fig. 6). Three patients in group A showed a marked increase, and the peak of activities was found around October. The other 2 patients also showed a gradual increase, but the change was slight. In group B, 1 patient (case 8) showed an increase in antithyroglobulin antibodies before the attack of allergic rhinitis, but 4 other patients showed no increase from December to September.

Compared to these changes, anti-streptolysin-O antibody levels did not change in any patient during the observation periods (fig. 7).

In order to examine the possibility of IgE-associated autoantibodies, sera with the highest anti-TSH-receptor antibody levels were obtained from cases 1, 4 and 5. Even after absorption of IgE, the activities of anti-TSH-receptor antibodies, anti-thyroid-peroxidase antibodies and antithyroglobulin antibodies were not changed (data not shown).

Serum levels of IL-5 and IL-13 were measured, but serum samples showed undetectable levels lower than the minimum detectable concentration.

Discussion

Little is known of the effect of allergic diseases on the clinical course of autoimmune disorders. It is often difficult to predict the time of association of a disease with an allergic disorder. Also many patients receive various kinds of immunosuppressive drugs. Therefore, the correct evaluation of spontaneous changes in disease activity is usually difficult. The prevalence of seasonal allergic rhinitis to Japanese cedar pollens is increasing in Japan, and more than 20% of subjects in the general population suffer from this allergy [19]. Japanese cedar pollen markedly disseminates at the end of February or early March every year, and thus the attacks of this pollinosis can be easily predicted. On the other hand, aggravation of Graves' disease is readily evaluated by changes in serum thyroid hormones and antithyroid antibody activities. For example, postpartum aggravation of autoimmune thyroid disease can be detected by postpartum development of thyroid dysfunction [20].

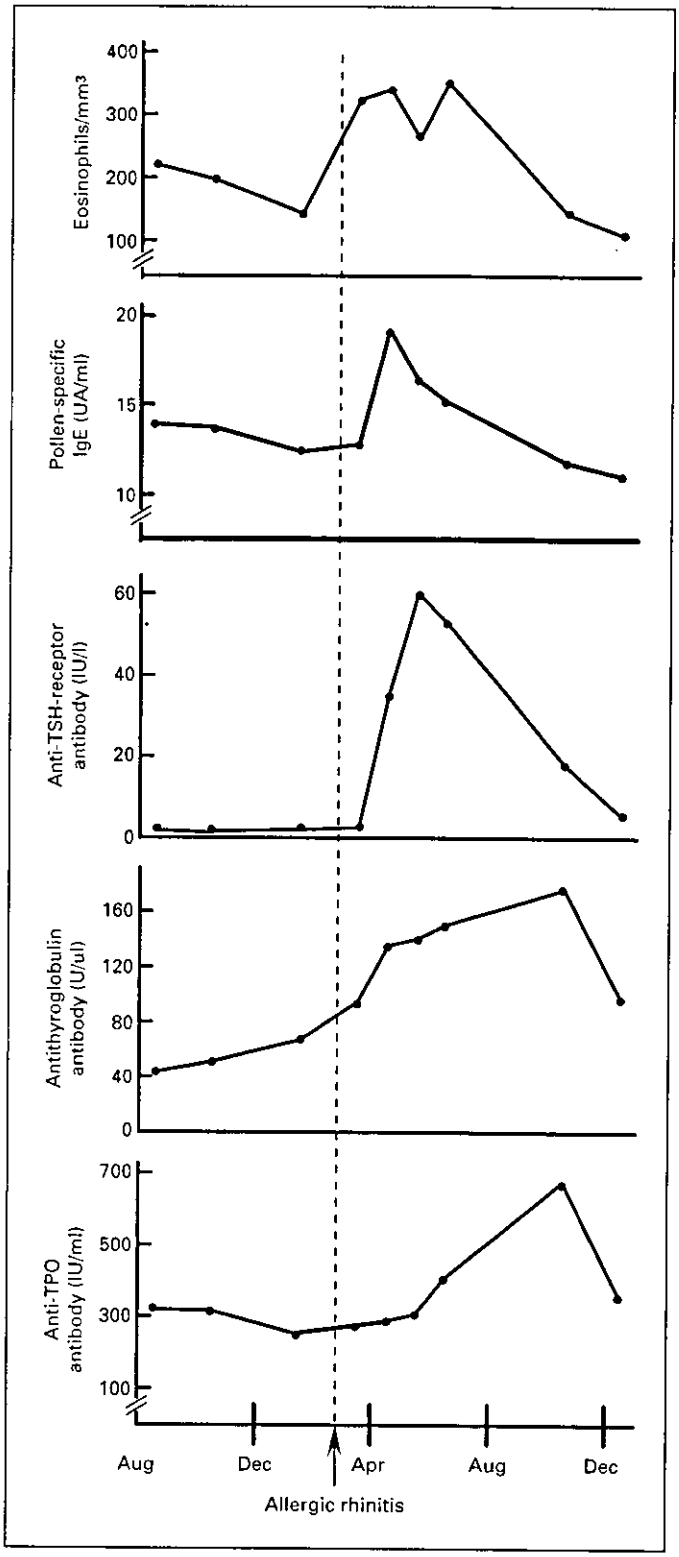
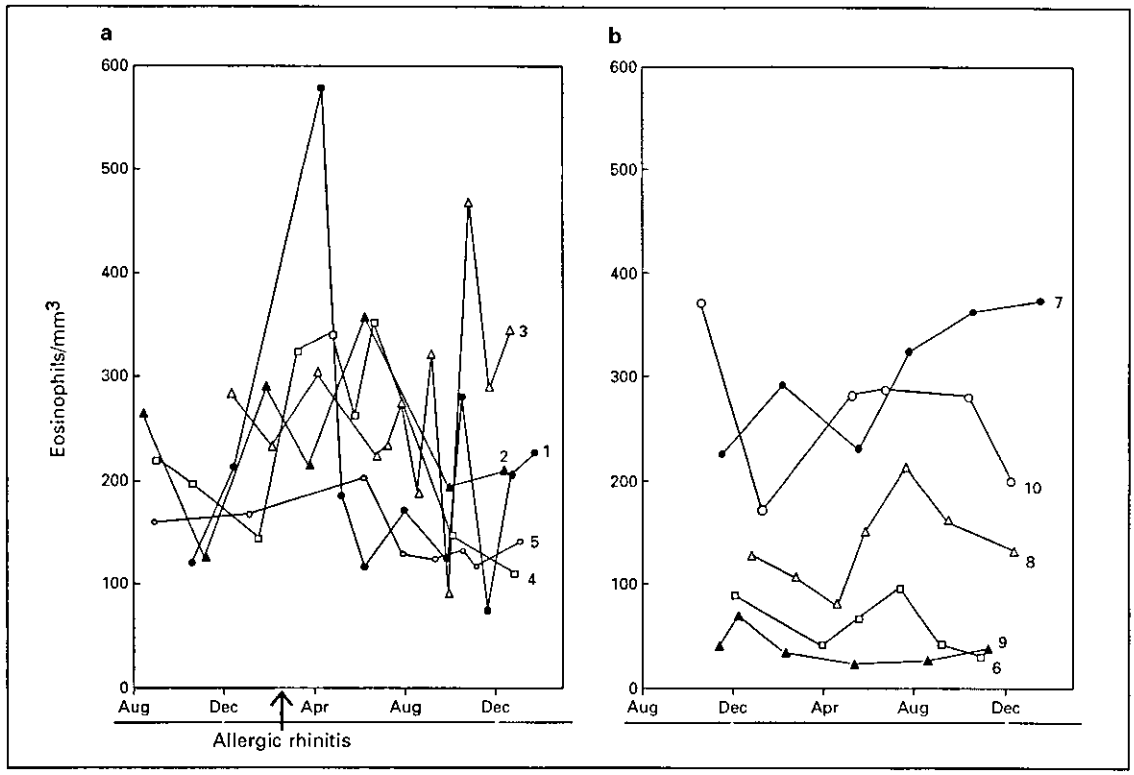


Fig. 1. Serial changes in peripheral eosinophil counts and serum levels of pollen-specific IgE, anti-TSH-receptor antibody, antithyroglobulin antibody and anti-thyroid-peroxidase (TPO) antibody in case 4.



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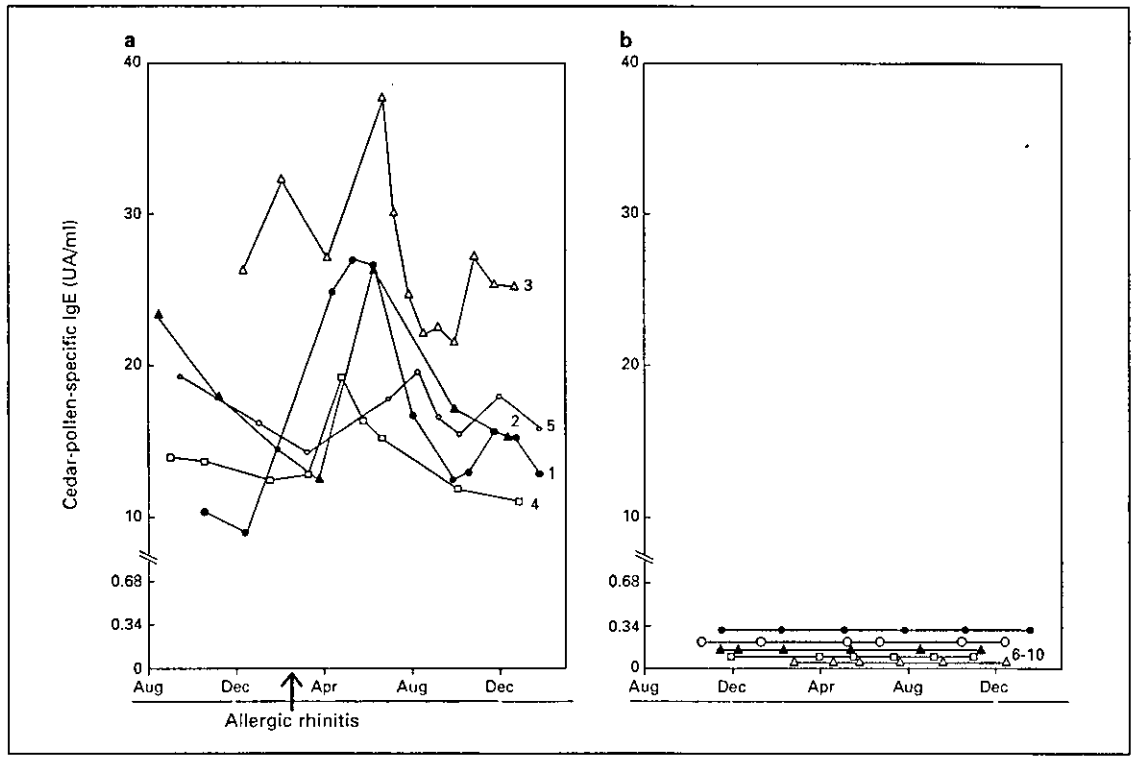

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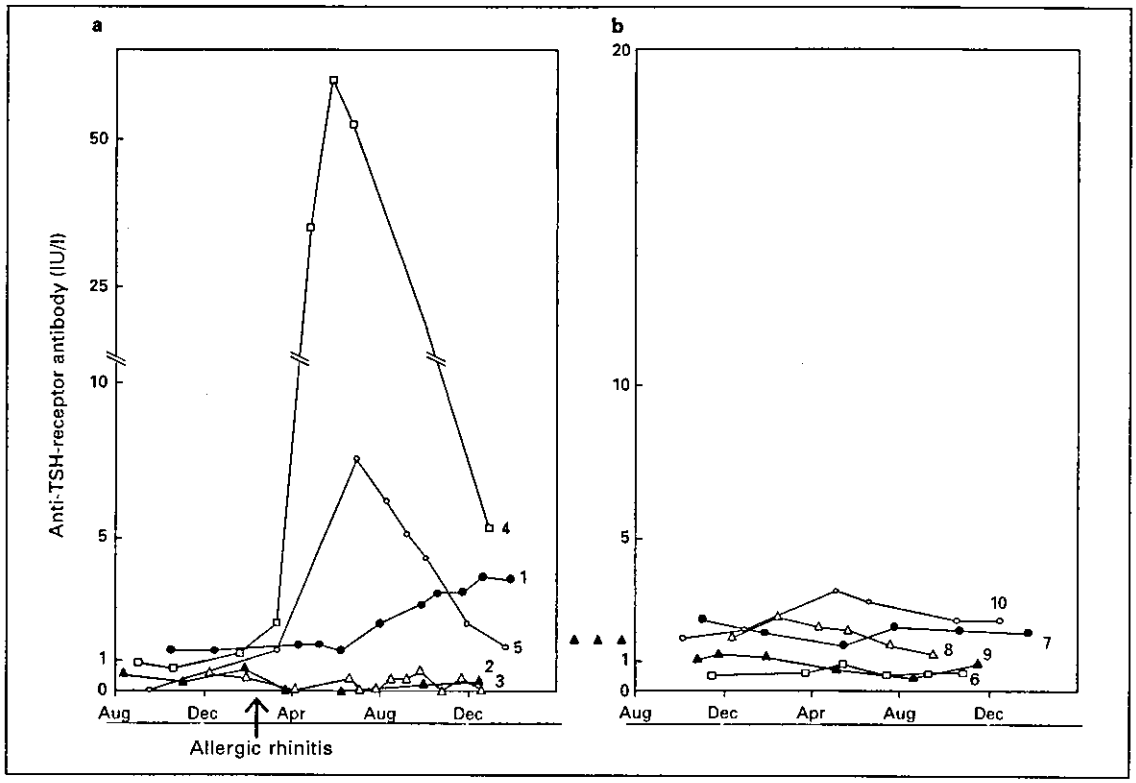
Fig. 2. Serial changes in peripheral eosinophil counts in patients with Graves' disease with (**a**) and without (**b**) seasonal allergic rhinitis.

Fig. 3. Serial changes in serum levels of cedar-pollen-specific IgE in patients with Graves' disease with (**a**) and without (**b**) seasonal allergic rhinitis.



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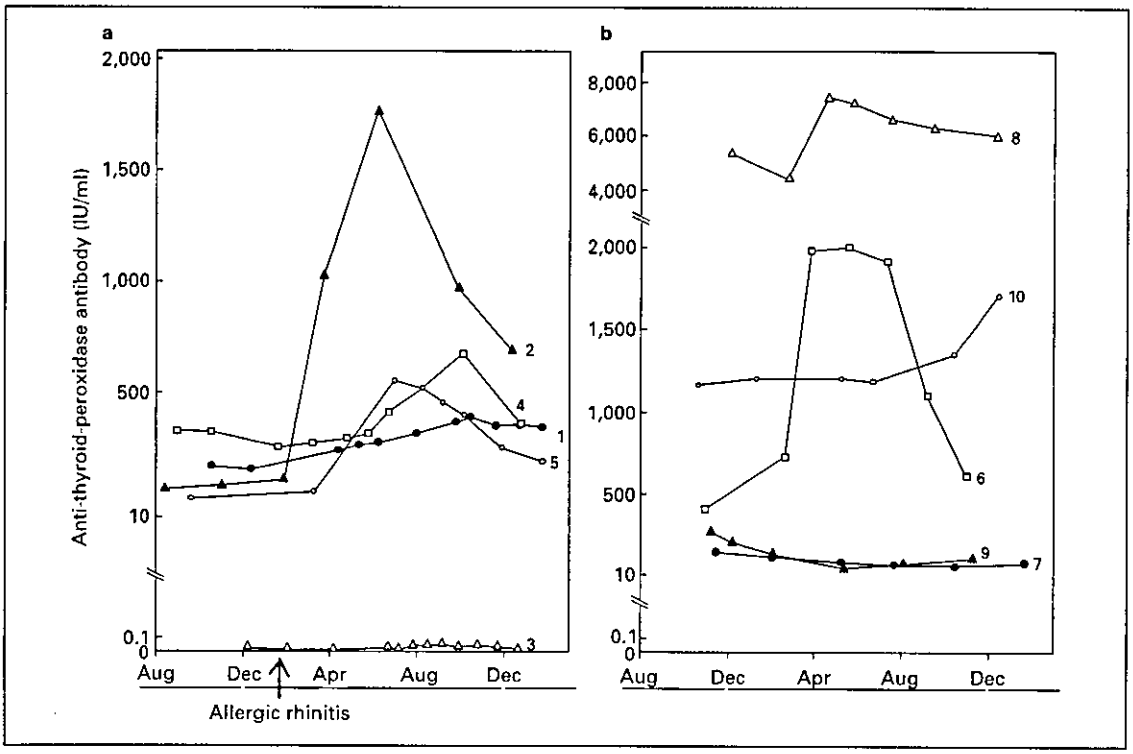

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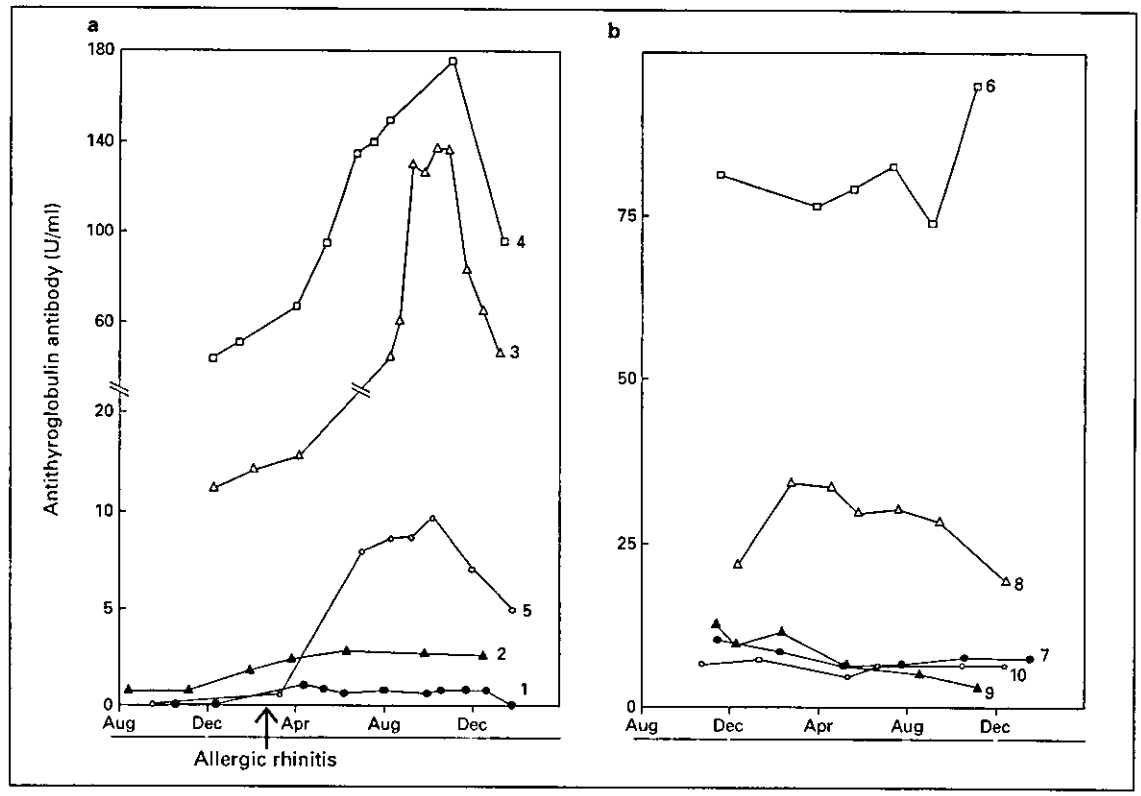
Fig. 4. Serial changes in serum levels of anti-TSH-receptor antibody in patients with Graves' disease with (**a**) and without (**b**) seasonal allergic rhinitis.

Fig. 5. Serial changes in serum levels of anti-thyroid-peroxidase antibody in patients with Graves' disease with (**a**) and without (**b**) seasonal allergic rhinitis.



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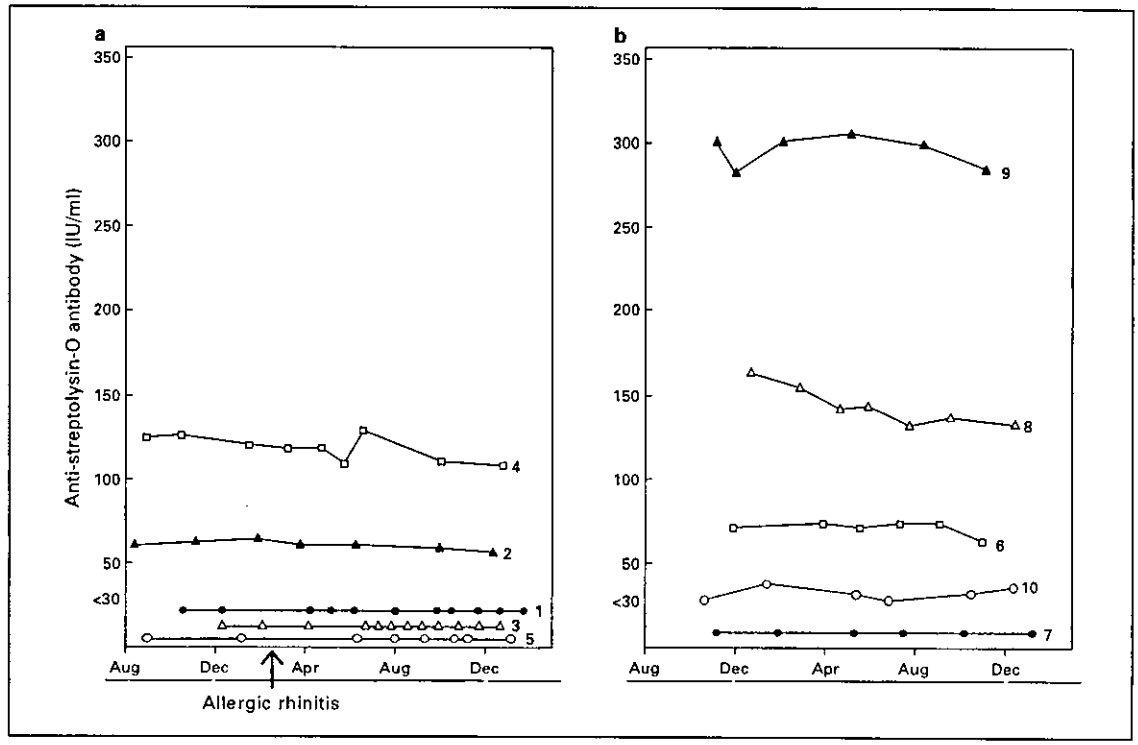

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Fig. 6. Serial changes in serum levels of antithyroglobulin antibody in patients with Graves' disease with (**a**) and without (**b**) seasonal allergic rhinitis.

Fig. 7. Serial changes in serum levels of anti-streptolysin-O antibodies in patients with Graves' disease with (**a**) and without (**b**) seasonal allergic rhinitis.

In this study, all patients with cedar pollinosis showed a clear increase in autoantibody titers as well as an increase in cedar-pollen-specific IgE activity. The drug effects on these changes were negligible, since the patients were receiving only minimal doses of methimazole or no medication, except for patient 5 who received a second course of 15 mg of methimazole in July due to relapse of thyrotoxicosis. Large doses of methimazole may have an immunosuppressive effect [21], but a minimal maintenance dose may not have such an influence. The natural seasonal influence could also be ruled out, since the control patients without pollinosis showed no significant change in the titers of any antibodies during the period of 18 months.

The increases in antibody activities started soon after the attacks of rhinitis for every antibody, but the peaks of activity were slightly different among these. The pattern of increase was similar between cedar-pollen-specific IgE and anti-TSH-receptor antibody, although 2 patients were always negative. It is interesting to speculate that B lymphocyte clones to produce antireceptor antibodies would be inactivated completely and reproduction of antireceptor antibodies could not be induced by allergic stimulation in the latter 2 patients. The peak of IgE and antireceptor antibody activities was around May–July. Compared to this, the peak was around October, that is 7 months after the attacks of rhinitis, for both anti-thyroid-peroxidase and antithyroglobulin antibodies, though the pattern was slightly different in individual patients. IgE antibodies, including that against cedar-pollen-specific IgE, bind to mast cells and basophils. Anti-TSH-receptor antibodies, which usually belong to IgG, bind to TSH receptors in the thyroid gland. On the other hand, antithyroglobulin and anti-thyroid-peroxidase antibodies have no such binding site, and thus consumption is different from the above 2 antibodies. These differences may be relevant to the differences of the patterns of antibody increase.

Even in group B, 2 patients (cases 6 and 8) showed an increase in anti-thyroid-peroxidase antibody in spring. In case 6, this antibody was increased before any attack of allergic rhinitis. An increase in antithyroglobulin antibodies was also observed in case 8 in spring, although it occurred before allergic rhinitis. They did not have concurrent respiratory disease in spring, and we do not know the reason why these 2 cases showed an antibody increase.

It is important to elucidate the mechanism of antibody increase. Allergic rhinitis is an inflammatory disease localized in the nose and eyes. If the involved lymph nodes draining the thyroid gland and mucosal respiratory tract are, at least in part, the same, some Th2-derived cytokines produced during allergen recognition can indirectly drive and amplify the ongoing autoantibody production by local B cells. IL-4, a Th2-associated cytokine, stimulates the production of IgG1, IgG3 and IgG4, as well as IgE [22]. Another Th2 cytokine, IL-13, stimulates IgE and IgG4 antibody production [23]. In this study, the increased antithyroid autoantibodies did not belong to the IgE fraction. It may be worthwhile to examine the IgG subclasses of these autoantibodies. Activation of a local Th2 immune reaction may stimulate systemic immunity toward Th2 predominance, but the measurement of circulating cytokines failed to prove this possibility.

In conclusion, local allergic reactions to environmental antigens in the nose and eyes may stimulate an immune reaction to Th2 predominance in the involved lymph node and may activate autoantibody production in the thyroid gland.

Acknowledgements

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References

1 Hidaka Y, Amino N, Iwatani Y, Itoh E, Matsunaga M, Tamaki H: Recurrence of thyrotoxicosis after attack of allergic rhinitis in patients with Graves' disease. J Clin Endocrinol Metab 1993;77:1667–1670.

2 Hidaka Y, Masai T, Sumizaki H, Takeoka K, Tada H, Amino N: Onset of Graves' thyrotoxicosis after an attack of allergic rhinitis. Thyroid 1996;6:349–351.

3 Weetman AP: Graves' disease. N Engl J Med 2000;343:1236–1248.

4 Izumi Y, Hidaka Y, Tada H, Takano T, Kashiwai T, Tatsumi KI, Ichihara K, Amino N: Simple and practical parameters for differentiation between destruction-induced thyrotoxicosis and Graves' thyrotoxicosis. Clin Endocrinol (Oxf) 2002;57:51–58.

5 Hidaka Y, Okumura M, Shimaoka Y, Takeoka K, Tada H, Amino N: Increased serum concentration of interleukin-5 in patients with Graves' disease and Hashimoto's thyroiditis. Thyroid 1998;8:235–239.

6 Okumura M, Hidaka Y, Kuroda S, Takeoka K, Tada H, Amino N: Increased serum concentration of soluble CD30 in patients with Graves' disease and Hashimoto's thyroiditis. J Clin Endocrinol Metab 1997;82:1757–1760.

7 Yamada T, Sato A, Komiya I, Nishimori T, Ito Y, Terao A, Eto S, Tanaka Y: An elevation of serum immunoglobulin E provides a new aspect of hyperthyroid Graves' disease. J Clin Endocrinol Metab 2000;85:2775–2778.

8 Hidaka Y, Kimura M, Izumi Y, Takano T, Tatsumi KI, Amino N: Increased serum concentration of eosinophil derived neurotoxin in patients with Graves' disease. Thyroid 2003;13:129–132.

9 Amino N, Hidaka Y, Takano T, Izumi Y, Tatsumi KI, Nakata Y: Association of seasonal allergic rhinitis is high in Graves' disease and low in painless thyroiditis. Thyroid 2003;13:811–814.

10 Rapoport B, McLachlan SM: Thyroid autoimmunity. J Clin Invest 2001;108:1253–1259.

11 Fisfalen ME, Palmer EM, Seventer GAV, Soltani K, Sawai Y, Kaplan E, Hidaka Y, Ober C, De Groot LJ: Thyrotropin-receptor and thyroid peroxidase-specific T cell clones and their cytokine profile in autoimmune thyroid disease. J Clin Endocrinol Metab 1997;82:3655–3663.

12 Aust G, Steinert SM, Lamesch P, Lohmann T: Graves' disease is associated with an altered CXCR3 and CCR5 expression in thyroid-derived compared to peripheral blood lymphocytes. Clin Exp Immunol 2002;127:479–485.

13 Romagnani P, Rotondi M, Lazzeri E, Lasagni L, Francalanci M, Buonamano A, Milani S, Vitti P, Chiovato L, Tonacchera M, Bellastella A, Serio M: Expression of IP-10/CXCL10 and MIG/CXCL9 in the thyroid and increased levels of IP-10/CXCL10 in the serum of patients with recent-onset Graves' disease. Am J Pathol 2002;161:195–206.

14 Petri M, Allbritton J: Antibiotic allergy in systemic lupus erythematosus: A case-control study. J Rheumatol 1992;19:265–269.

15 Sequeira JF, Cesic D, Keser G, Bukelica M, Karanagnostis S, Khamashta MA, Hughes GR: Allergic disorders in systemic lupus erythematosus. Lupus 1993;2:187–191.

16 Shahar E, Lorber M: Allergy and SLE: Common and variable. Isr J Med Sci 1997;33:147–149.

17 Tanihara S, Oki I, Ojima T, Nakamura Y, Yanagawa H: Process and current status of the epidemiologic studies on cedar pollinosis in Japan. J Epidemiol 1999;9:20–26.

18 Maugendre D, Massart C: Clinical value of a new TSH binding inhibitory activity assay using human TSH receptors in the follow-up of antithyroid drug treated Graves' disease: Comparison with thyroid stimulating antibody bioassay. Clin Endocrinol (Oxf) 2001;54:89–96.

19 Sakurai Y, Nakamura K, Teruya K, Shimada N, Umeda T, Tanaka H, Yoshizawa N: Prevalence and risk factors of allergic rhinitis and cedar pollinosis among Japanese men. Prev Med 1998;27:617–622.

20 Amino N, Mori H, Iwatani Y, Tanizawa O, Kawashima M, Tsuge I, Ibaragi K, Kumahara Y, Miyai K: High prevalence of transient postpartum thyrotoxicosis and hypothyroidism. N Engl J Med 1982;306:849–852.

21 Weetman AP, Holt ME, Campbell AK, Hall R, McGregor AM: Methimazole and generation of oxygen radicals by monocytes: Potential role in immunosuppression. Br Med J 1984;288:518–520.

22 Jenmalm MC, Bjorksten B, Macaubas C, Holt BJ, Smallacombe TB, Holt PG: Allergen-induced cytokine secretion in relation to atopic symptoms and immunoglobulin E and immunoglobulin G subclass antibody responses. Pediatr Allergy Immunol 1999;10:168–177.

23 Punnonen J, Aversa G, Cocks BG, McKenzie AN, Menon S, Zurawski G, de Waal Malefyt R, de Vries JE: Interleukin 13 induces interleukin 4-independent IgG4 and IgE synthesis and CD23 expression by human B cells. Proc Natl Acad Sci USA 1993;90:3730–3734.

Generation of a transgenic animal model of hyperthyroid Graves' disease

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Graves' disease (GD) is an organ-specific autoimmune disease characterized by hyperthyroidism. Agonistic anti-thyrotropin receptor antibodies (thyroid-stimulating antibodies, TSAb), which mimic the thyrotropin (TSH) action, are thought to cause GD. The precise immunological mechanism of TSAb production, however, remains elusive. Previous immunization approaches using TSH receptor led to transient hyperthyroidism, but did not seem sufficient for comprehensive understanding of the development of autoimmune responses. To create GD-related autoimmunity in mice, we here generated TSAb-transgenic mice in which a patient-derived TSAb is expressed in B cells. Expression of the human TSAb in mice resulted in various manifestations of hyperthyroidism including increased free thyroxine levels with concomitantly decreased TSH levels, increased thyroid uptake of technetium pertechnetate, hyperthermia and thyroid hyperplasia. We found a correlation between the serum levels of human TSAb immunoglobulin and free thyroxine. In addition, conventional B cells expressing the TSAb were partially deleted in the periphery while B1 cells expressing the TSAb persisted and accumulated in the peritoneal cavity, a finding consistent with previous demonstrations that the maintenance of B1 cells plays an important role in the development of autoimmune diseases. Thus, our transgenic mouse may provide a novel and useful animal model for elucidating the pathogenesis and pathophysiology of GD.

Key words: Graves' disease / Animal model / Transgenic mouse / Anti-TSH receptor antibody / Immunoglobulin gene

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1 Introduction

Graves' disease (GD) is a common autoimmune disease characterized by the production of antibodies (Ab) against thyrotropin (TSH) receptor (TSHR) [1–4]. Agonistic anti-TSHR Ab, or thyroid-stimulating Ab (TSAb), mimic TSH function and induce the overproduction of thyroid hormones. GD patients manifest various symptoms of hyperthyroidism including increased heart rate, excess perspiration and weight loss. TSAb can be detected in the serum of more than 90% of newly diagnosed GD patients with hyperthyroidism [3]. The measurement of TSAb in patients with GD is a useful predictor of relapse and remission [5].

A GD animal model will clarify the mechanisms underlying the development of GD including that of TSAb production. Recently, two attempts were made by immunizing mice using human TSHR: one by the injection of TSHR-transfected cells simultaneously expressing MHC

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Abbreviations: GD: Graves' disease **TSH:** Thyrotropin **TSAb:** Thyroid-stimulating antibody **FT4:** Free thyroxine **Tg:** Transgenic **hIgM:** Human IgM **SP:** Spleen **PerC:** Peritoneal cavity **TPT:** Technetium pertechnetate **BBT:** Basal body temperature

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class II [6]; the other by genetic immunization using TSHR cDNA [7]. Although these immunized mice showed some typical phenotypes associated with hyperthyroidism, the responses were temporary and TSAb were produced against non-self TSHR. The process of autoantibody production in these mice presumably lacks the persistent abrogation of self tolerance.

To circumvent the lack of an appropriate animal model for the study of GD, we undertook a different approach. In previous studies, we established a B cell clone producing a TSAb from a GD patient [8, 9]. We subsequently demonstrated that this TSAb bound to TSHR and stimulated the thyroid *in vitro* [8, 10] and *in vivo* [11]. These observations prompted us to generate a transgenic (Tg) mouse constitutively expressing the human TSAb. Since B cells expressing the TSAb are subjected to the host immune system, we postulated that these TSAb-Tg mice might develop immunological responses associated with autoimmunity, such as clonal deletion, anergy or the breakage of self tolerance. Indeed, we demonstrate in this report that these TSAb-Tg mice manifest many of the clinical features of hyperthyroidism and develop immunological responses against B cells that produce autoantibodies. These TSAb-Tg mice provide a novel and useful model for the study of endocrinological and immunological aspects of GD. In particular, studies concerning the disruption of self tolerance, a process which is responsible for the TSAb production, will be greatly facilitated.

2 Results

2.1 Establishment of transgenic mice expressing a patient TSAb

To express an agonistic TSAb in mice, we designed a transgene construct (Fig. 1A). A 5' flanking region of the original human Ig (TSAb) gene [8] was used as a provisional promoter and a human Cμ segment was used for the constant region of H chain. We established two founder lines; one carried approximately ten copies of the transgene on chromosome (Ch.) 10 while the other carried ~20 copies on a Ch. 7–Ch. 9 translocation (data not shown). As no phenotypic differences between these two lines were observed, we show combined results from both lines in the following studies.

2.2 B cell surface expression of human TSAb in TSAb-Tg mice

To determine whether the TSAb was expressed in the TSAb-Tg mice, we quantified serum human IgM (hIgM)

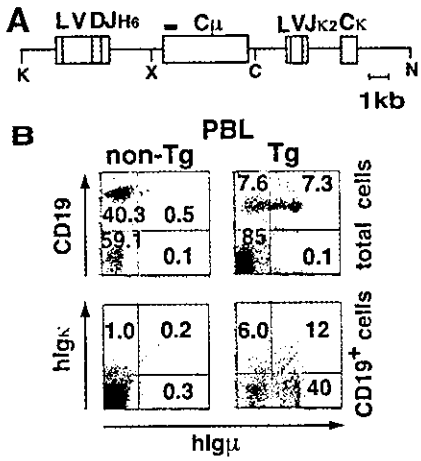

Fig. 1. Schematic design of the transgene and the expression of hIgM on B cells in TSAb-Tg mice. (A) The transgene. Closed box indicates the probe used for Southern blot analysis. Relevant restriction sites are shown: K, KpnI; X, XhoI; C, ClaI; N, NotI. (B) HIgM and Igκ expression on PBL from Tg mice. Three-color flow cytometry using anti-CD19, anti-human IgM and anti-Igκ Ab was performed. Numbers represent the percentage of a particular subset.

by ELISA. Average levels of serum hIgM in TSAb-Tg mice were 47±38 μg/ml (mean ± SD, n=74), while transgene-negative littermates expressed undetectable levels of hIgM, thus confirming that the transgene was successfully expressed in the Tg mouse. Next we examined TSAb-Tg B cells for expression of surface hIgM. As shown in Fig. 1B, 12 % of B cells from the Tg mouse were $hIgM^+/hIg\kappa^+$. Moreover, total B cell numbers in the TSAb-Tg mice were much lower than expected, suggesting that B cells can be autoreactive, and clonal deletion, one of the key mechanisms for self tolerance, may have occurred (see below). Expression of the transgene was not observed in T cells (Fig. 1B) or other tissues (data not shown).

2.3 Development of hyperthyroidism in TSAb mice

Thyroid function in TSAb-Tg mice was assessed by measuring serum free thyroxine (FT4) and TSH levels. The FT4 levels of TSAb-Tg mice were significantly higher than those of non-Tg littermates (mean ± SD: Tg: 27.5±8.0 pmol/l, n=74 *vs.* non-Tg: 14.9±3.9 pmol/l, n=38, p<0.001) (Fig. 2A), while the TSH levels of Tg mice (mean ± SD: 1.3±1.2 ng/ml) were lower than those of non-Tg littermates (mean ± SD: 4.4±2.2 ng/ml) (Fig. 2B). Based on the thyroid function of non-Tg mice, we arbitrarily defined the hyperthyroid status as follows: FT4 >22.7 pmol/l (mean + 2 SD) and TSH <1.4 ng/ml (mean

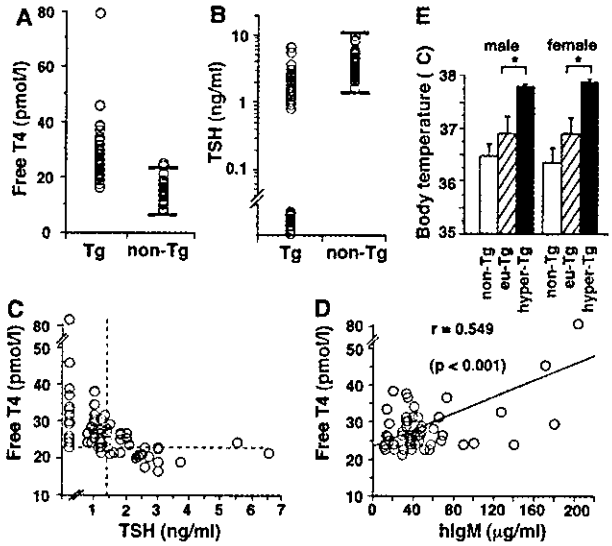

Fig. 2. The human TSAb-Tg mice manifested hyperthyroidism. (A) Serum FT4 levels of Tg (n=74) and non-Tg (n=38) mice are shown. Horizontal bars in the non-Tg column represent the mean ± 2 SD boundaries. (B) TSH levels of the same mice as in (A). To calculate the deviation range (mean ± 2 SD) for non-Tg mice, values were normalized following logarithmic transformation. (C) Correlation of FT4 and TSH levels in each individual Tg mouse as in (A) and (B). Horizontal broken line corresponds to the upper bar level (mean + 2 SD) shown in (A), and vertical broken line to the lower bar level (mean – 2 SD) shown in (B). Mice in the upper left quadrant (n=50) satisfy the criteria for hyperthyroidism. (D) Correlation of FT4 and hIgM levels in individual Tg mice with low TSH levels (n=60). r, correlation coefficient; using Person's correlation coefficient test. (E) BBT measurements. Body temperatures of non-Tg (open box; male, n=6; female: n=7), euthyroid Tg (hatched box; male, n=7; female, n=4) and hyperthyroid Tg (closed box; male, n=6; female, n=3) mice are shown. The mean ± SE values are shown for each set of data; *p<0.05.

– 2 SD, using normalized logarithmic transformation). Fifty of 74 TSAb-Tg mice (68%) met this criterion (Fig. 2C). Of them, 24 mice had undetectable levels of TSH (<0.1 ng/ml). Thirteen mice that displayed increased FT4 but normal TSH levels. In these mice, the duration or degree of the increased FT4 levels might not be sufficient to suppress TSH levels. In the majority of TSAb-Tg mice examined, we observed positive correlations between serum hIgM and FT4 levels (Fig. 2D). These results indicate that the hIgM (TSAb) is indeed pathogenic, and that its level determines the severity of hyperthyroidism in the Tg mice.

2.4 Clinical manifestations of hyperthyroidism in TSAb-Tg mice

Thyroid hormones stimulate various metabolic processes, including energy expenditure and the metabolism of various nutrients, and thus induce increased body temperature as one of the clinical manifestations of thyrotoxicosis [12]. We observed higher basal body temperature (BBT) in both male and female hyperthyroid Tg mice compared to euthyroid Tg mice. There was no significant difference in the BBT between euthyroid Tg and non-Tg mice (Fig. 2E). These findings strongly suggest that thyroid function influences BBT in TSAb-Tg mice. In addition, hyperthyroid TSAb-Tg mice showed higher activity than euthyroid Tg mice as measured by distance moved in a given time scale (data not shown).

2.5 Increased thyroid technetium uptake by hyperthyroid Tg mice

Technetium pertechnetate (TPT) scintigraphy was performed to confirm that the hyperthyroidism in the TSAb-Tg mouse was caused by hyperactivity of the thyroid gland, not by destructive thyroiditis. The pertechnetate ion is transported into thyroid tissue by an iodide-concentrating mechanism but has a shorter half-life (6 h) than other radioiodines (^{131}I: 8 days; ^{123}I: 13 h). We injected mice with TPT via the tail vein and assessed the time course of TPT transport into the thyroid. In non-Tg mice, TPT was predominantly found in the stomach though trace amounts were present in the thyroid (Fig. 3A). Thyroidal TPT accumulation slightly increased up to 9% (mean ± SD: 7.8±0.64%) and saturated at 90–120 min (Fig. 3B) post-injection. In contrast, hyperthyroid TSAb-Tg mice accumulated TPT up to significantly higher levels (mean ± SD: 16.5±3.1%) and sustained this for at least 150 min. These results demonstrate that iodide transport is substantially increased in the hyperthyroid TSAb-Tg mice.

2.6 Hyperplastic thyroid in hyperthyroid mice

Thyroid hyperplasia is a typical characteristic of GD. Histological examinations of the thyroids from hyperthyroid TSAb-Tg mice revealed irregular-sized follicles with diffuse hypercellularity (Fig. 3C). In large follicles with large nuclei, vacuoles due to hypersecretion were seen in the boundary of epithelial cells; richness of interstitial arterioles was also seen. These results are consistent with hyperthyroxinemia and high thyroid technetium uptake. Several accompanied features of GD were not detectable in our hyperthyroid TSAb-Tg mice, such as lymphocyte aggregates in the thyroid or evidence of muscle destruction associated with ophthalmopathy.

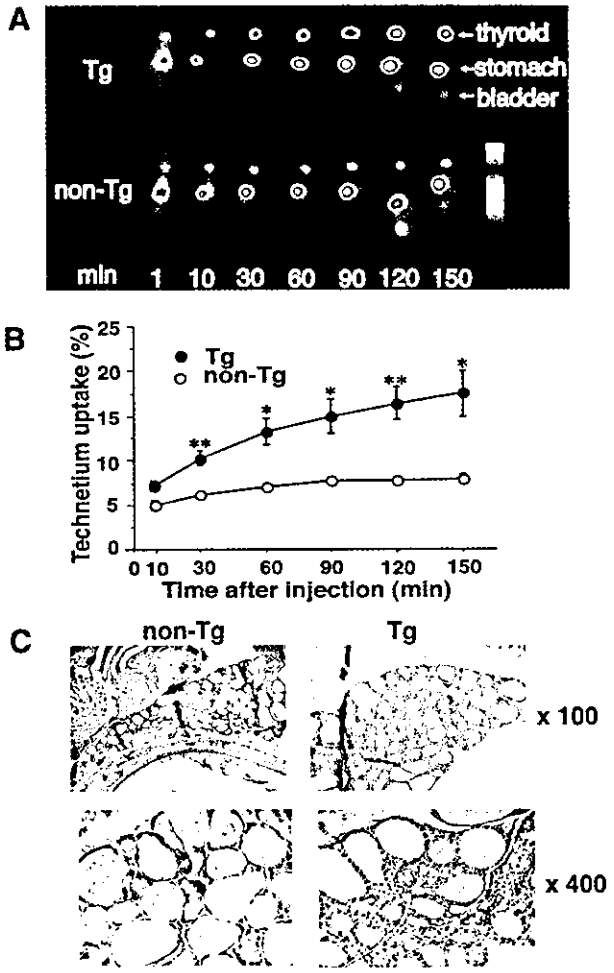

Fig. 3. High technetium uptake and hyperplasia in the thyroid of the human TSAb-Tg mouse. (A) Technetium uptake planar images comparing the Tg and non-Tg mice. Images were taken at 1, 10, 30, 60, 120 and 150 min after injection of 2 mCi TPT into the tail vein. Striped color bar indicates arbitral intensity of the accumulated radioactivity. The white-red end indicates higher accumulation. (B) Time course of technetium uptake in the Tg (closed circle; n=3) and non-Tg (open circle; n=3) mice. The mean ± SE values are shown for individual time points; $^{*}p<0.05$, $^{**}p<0.01$, Student's t-test. (C) Thyroid gland histology. Magnification factors: top, ×100; bottom, ×400. Compared to the normal tissue, the thyroid gland from the Tg mouse displays diffuse hypercellularity and follicles with irregular sizes. Note that small cells are surrounded by square epithelial cells and large ones are surrounded by taller rounded cells.

2.7 Clonal deletion of TSAb-positive B cells in peripheral lymphoid organs

As described earlier, B cell numbers in the TSAb-Tg mice were much lower than expected, suggesting that autoreactive TSAb-positive B cells might have been partially eliminated by clonal deletion. To test this hypothesis, B cells from bone marrow (BM), spleen (SP) and peritoneal cavity (PerC) of TSAb-Tg mice were analyzed. The percentages of hIgM$^+$/hIgκ$^+$ B cells in the whole lymphoid cells from the SP and BM of Tg mice were similar to those in the peripheral blood lymphocytes (PBL) (Fig. 4A). Total B cell numbers decreased in the SP and BM as well as in PBL (Fig. 4B). Statistical analysis confirmed that the total B cell numbers in TSAb-Tg PBL were significantly decreased compared with that of non-Tg littermates. However, percentages of hIgM$^+$ B cells in the PBL and PerC of TSAb-Tg mice varied among mice. These results suggest that clonal deletion of TSAb-bearing B cells may be occurring in the TSAb-Tg mice.

2.8 B cells that survive clonal deletion accumulate in the PerC

If clonal deletion regulates the numbers of TSAb-bearing B cells in peripheral lymphoid organs, such cells may survive deletion in places where the immunological surveillance mechanism does not operate efficiently. Thus we investigated the number and composition of B cells in the PerC. We observed an increase of B cells in the PerC of the TSAb-Tg mice compared with that of non-Tg littermates (Fig. 4B); and the percentages of B cells expressing hIgM and Igκ in the PerC were much greater than those in the PBL (Fig. 4A). Further analysis of B cells in PerC using anti-Mac-1 Ab revealed that the substantial number of Mac-1$^+$ B1 cells was present and that approximately 40% of B1 cells expressed hIgM (Fig. 4C). Furthermore, most of hIgM$^+$ B cells were Mac-1$^+$. Of hIgM$^+$ B cells, the percentages of B1a (CD5$^+$) and B1b (CD5$^-$) cells were almost equal. These results are consistent with the previous ones that B cells in the PerC play an important role in autoimmunity [13].

2.9 Oral LPS administration induces hyperthyroidism in the TSAb-Tg mice

We examined whether administration of LPS induce the hyperthyroid state in the euthyroid TSAb-Tg mice. Before LPS administration, mean ± SD of serum FT4 and TSH levels of euthyroid TSAb-Tg mice were 11.4±1.15 pmol/ml and 3.8±0.64 ng/ml (non-Tg mice: 10.9±0.07 pmol/ml and 3.91±0.39 ng/ml), respectively. Seven days after oral administration of 10 μg LPS, their levels changed to 24.6±1.03 pmol/ml (n=5, $p<0.0001$) and 0.07±0.23 ng/ml (n=5, $p<0.01$) (non-Tg mice: 9.4±0.09 pmol/ml and 5.1±0.12 ng/ml), respectively (Table 1). These data suggest that the LPS administration activated TSAb-producing B or B1 cells in the Tg mice.

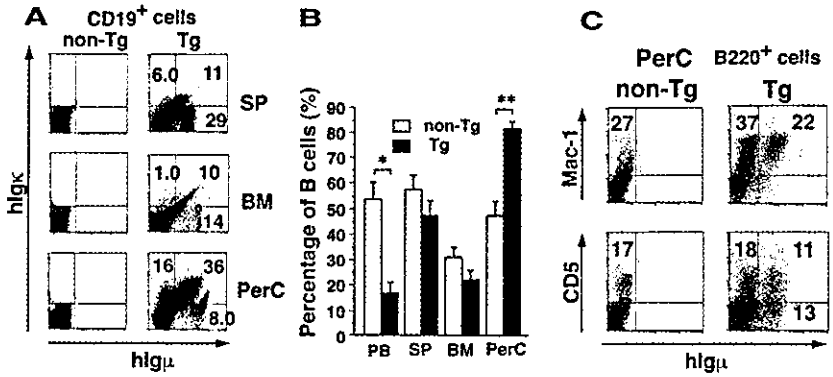

Fig. 4. Clonal deletion and survival of TSAb-bearing B cells in the Tg mouse. (A) Flow cytometry of hIgM expression on the B cells of SP, BM and PerC. Cells were stained as described in Fig. 1B. (B) The B cell compartment size in lymphoid organs (open box: non-Tg, n=3; closed box: Tg, n=3). Data are means ± SE; (*p<0.05, **p<0.01, Student's t-test for unpaired data. (C) Flow cytometry of B1 cells in PerC.

3 Discussion

In this study, we generated hyperthyroidism in mice by expressing a patient-derived anti-TSHR Ab as a transgene. We were particularly interested in determining whether this strategy would evoke in mice similar autoimmune responses to those that are commonly seen in humans with GD. The human TSAb was successfully expressed in our Tg mice, and stimulated thyroid cells to produce excessive amounts of thyroid hormones (FT4). Consequently, these TSAb-Tg mice developed a series of hyperthyroidism symptoms typically seen in GD, which include increased serum FT4 levels concomitant with suppressed or decreased TSH levels, hyperthermia, hyperactivity, and an increase in body temperature. In addition, thyroid cells in these mice showed increased technetium uptake and displayed hyperplastic changes, all of which are typically seen in GD.

Table 1. Hyperthyroidism induction by oral LPS administration[a)]

	FT4 (pmol/l)	TSH (ng/ml)
TSAb-mice		
before LPS	11.4±1.15	3.80±0.64
after LPS	24.6±1.03*	0.77±0.23*
non-Tg mice		
before LPS	10.9±0.07	3.91±0.39
after LPS	9.4±0.09	5.1±0.12

[a)] Values are expressed as mean ± SE (*p<0.01, n=5, *vs.* "before LPS").

We took a new approach in generating a GD animal model. In the previous models, mice were immunized with human TSHR, which resulted in a transient autoimmune response. The non-persistent nature of the autoimmune response in the immunized mice is, supposedly, a consequence of the transient expression of the nominal Ag. However, the continuous presence of the Ag could induce tolerance, rather than leading to a persistent autoimmune response. To circumvent this problem, we persistently expressed the autoantibody, derived from a GD patient, in our Tg mouse. Indeed, many of the pathological and clinical changes associated with GD appeared in our TSAb-Tg mice. This is the first mouse model in which the constitutive expression of a human autoantibody has resulted in the successful development of an autoimmune disease.

Using this mouse model, we investigated the immunological factors which influence the development of hyperthyroidism in mice. The serum FT4 levels, a direct indicator of hyperthyroidism, strongly correlated with the serum hIgM levels. Nevertheless, the numbers of total B cells in PBL, SP and BM were significantly lower in the Tg mice compared to those of non-Tg littermates. Moreover, the number of Tg B cells variably decreased in the PBL, BM and SP in Tg mice and did not show correlation with serum hIgM or FT4 levels. These pieces of evidence suggest that a deletion process against the self-reactive B cells took place but the deletion of Tg B cells in the periphery did not abrogate the production of autoreactive TSAb in the Tg mice. Interestingly, we observed that the number of total B cells dramatically increased in the PerC of the Tg mice and that many of those B cells expressed hIgM (TSAb). Collectively, these findings suggest that B cells in PerC, rather than those in the periphery, are implicated in the development of GD in the Tg mice.

Similar observation was obtained from studies using a Tg model of autoimmune hemolytic anemia (AIHA), in which the mouse anti-erythrocyte autoantibody was expressed as a transgene [13, 14]. Using this model, Honjo and co-workers demonstrated the importance of a subset of B cells, namely B1 cells [15, 16], in the development of AIHA. These investigators further demonstrated that B1 cells which had survived clonal deletion accumulated in the PerC, and that these B1 cells could produce autoantibodies following LPS, IL-5 or IL-10 administration [17, 18]. In contrast, conventional B cells, even in the PerC as well as in the periphery, were deleted in this model. These findings, combined with our own observation, lead us to speculate that B cells in PerC can produce the TSAb, causing the development of hyperthyroidism in our Tg mice.

Administration of LPS into the Tg mice resulted in the elevated serum FT4 levels. Consistently, clinical studies demonstrated that the increase of $CD5^+$ B cells, though in the PBL compartment, correlates well with the disease activity in GD patients [19, 20]. Interestingly, this fact correlates with the data that the number of $CD5^+$ B cells in the PerC tends to decrease; on the other hand, those in PB tend to increase when hyperthyroidism was induced by LPS administration. However, we did not determine the number of plasma cells, a population presumably secreting the TSAb, in these compartments (*i.e.* periphery and PerC). Moreover, a role of T cells has not yet been studied in the present model. In addition to B cell dysregulation, intrinsic T cell dysfunction may be involved in chronic autoimmunity [21, 22]. Therefore, it is not clear how the present model reflects T cell help and dependency of the chronic Ab production on innate stimuli in the autoimmune response. For example, T cell-driven B2 differentiation into memory plasma cells has not been ruled out as a possible pathological mechanism in GD. Thus future investigation is needed to address these issues.

We noticed one difference between the AIHA and TSAb-Tg mice in addition to the similar accumulation of B1 cells in PerC and the deletion of peripheral B cells. Both B1 and B2 cells in TSAb-Tg mice increased in number in the PerC (data not shown), whereas only B1 cells but not B2 cells increased in AIHA mice. This difference may be associated with the distribution of the nominal Ag in each system. TSHR is widely expressed by the adipose tissue, thymus, kidney, heart and brain, in addition to the thyroid [23, 24], while erythrocytes are seen only in the blood. Such difference in Ag distribution could supposedly lead to different consequences in the development of autoimmunity in each animal model because autoreactive B cells might encounter their Ag at different locations and/or stages in their development. However, both animal models manifested the development of autoimmune responses, suggesting that the distribution of Ag did not meaningfully affect the onset of the immune responses in these animal models.

Most TSAb detected in patients with GD belong to the IgG class [1, 25]. In fact, clone B6B7, which was isolated from PBL of a GD patient, is originally the IgG clone and has a significant number of somatic mutations in V region genes of H and L chains, indicating the involvement of somatic mutations for the TSAb specificity [8, 9]. Although the isotype was changed from IgG to IgM in this TSAb-Tg mouse, this would not influence the binding affinity to TSHR or TSAb activity *per se*. Furthermore, we found IgM TSAb from PBL of GD patients and indicated the affinity maturation of TSAb driven by Ag in IgM-producing lymphocytes [8, 26]. The isotype alteration, however, might affect on effector functions, *i.e.* complement fixation. This issue should be further investigated in the future study.

Clinical studies implicated the involvement of environmental factors in the development of GD in humans. We observed, as noted above, that an oral administration of LPS into the Tg mice caused an elevation of serum FT4 levels suggesting that the activation of B cells in PerC may augment the TSAb production in the Tg mice. Furthermore, this observation implies that certain bacterial infection in humans, through the activation of B1 cells in PerC, may episodically trigger or augment autoantibody production in human GD patients.

In summary, we developed a Tg mouse model of GD. These mice should provide us with unique opportunities not only to study the pathogenesis of GD but also to develop a new treatment strategy for this autoimmune disease.

4 Materials and methods

4.1 DNA construction

We isolated the V region cDNA of both H and L chains of the TSAb from an EBV-transformed B cell clone, B6B7, obtained from PBL of a patient with GD [8, 9]. The characteristics of B6B7 were described previously [8–11, 27]. Next, three PCR-amplified DNA fragments were tandemly ligated: the 5.7-kb L chain, the 3.7-kb VDJ fragment containing 5' non-coding region and the 4.7-kb constant fragment of H chain (Fig. 1A). A 500-bp fragment upstream of the ATG initiation site was cloned as a putative promoter. An intron enhancer derived from the H chain and an intron enhancer/matrix attachment region of κ chain were also included. Cμ segment was used for the constant region of H chain,

because the isotype change from IgG to IgM will not influence the activity.

4.2 Generation of transgenic mice

A 14.1-kb transgene was microinjected into fertilized eggs of C57BL/6J mice. We detected the transgene integration by Southern blot analyses with the probe (Fig. 1A). Mice were maintained in conventional but not pathogen-free conditions and analyzed at 12–20 weeks of age. All subsequent animal studies were conducted based on a guideline approved by the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University.

4.3 Flow cytometry

Lymphocytes from peripheral blood, BM, SP and PerC were analyzed by flow cytometry on a FACSCalibur™ (Becton Dickinson); anti-mouse CD19-biotin, anti-mouse B220-APC, anti-mouse CD5-PE and anti-mouse Mac-1-PE Ab were purchased from PharMingen. Goat anti-human μ-FITC and anti-human κL chain-PE were from Biosource Int. Streptavidin-PE-Cy5 was from DAKO.

4.4 ELISA system

The levels of secreted hIgM and hIgκ were measured by ELISA using goat anti-hIgM or anti-hIgκ Ab as the coating Ab, and alkaline phosphatase-conjugated anti-hIgM or anti-hIgκ Ab as the secondary Ab. All Ab were from Biosource.

4.5 Thyroid function

Serum FT4 and TSH concentrations were measured using a commercial kit (ENZAPLATE FT4, Bayer Medical; rat TSH ^{125}I assay, Amersham Pharmacia Biotech).

4.6 Scintigraphy

Imaging was conducted using a scintigraphic gamma camera with a parallel collimator. TPT ($^{99m}TcO_4^-$; Mediphysics; 2 mCi/0.1 ml) was injected into the tail vein of anesthetized mice and the radioactivity accumulated in the thyroid was measured. The uptake ratio was determined by measuring the accumulated radioactivity in the thyroid shaped by region of interest subtracting the radioactivity found in other parts of the body as background, and dividing by the total injected radioactivity. TPT decrement radioactivity was corrected as follows: A (corrected activity) = A' (counted activity) × power (1/2, min/6.02 × 60 min).

4.7 Body temperature

We measured the anal temperature of mice at 7:00 p.m. for four consecutive days to minimize the body temperature fluctuation related to the estrous cycle.

4.8 Histology of thyroid and retro-orbital tissue

Thyroid glands and orbital tissue with the eyeball were placed in formalin. Five-micrometer-thick paraffin-embedded sections from each tissue were stained with hematoxylin-eosin.

4.9 Lipopolysaccharide administration

One-hundred micrograms LPS (Sigma Chemical Co., St. Louis, MO) dissolved in 500 μl was orally administered to the mice through a polyethylene tube of 1 mm diameter.

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References

1 **Rees Smith, B., McLachlan, S. M. and Furmaniak, J.,** Autoantibodies to the thyrotropin receptor. *Endocr. Rev.* 1988. **9:** 106–121.

2 **DeGroot, L. J. and Quintans, J.,** The causes of autoimmune thyroid disease. *Endocr. Rev.* 1989. **10:** 537–562.

3 **Weetman, A. P.,** Graves' disease. *N. Engl. J. Med.* 2000. **343:** 1236–1248.

4 **Akamizu, T.,** Antithyrotropin receptor antibody: an update. *Thyroid* 2001. **11:** 1123–1134.

5 **Davies, T. F., Roti, E., Braverman, L. E. and DeGroot, L. J.,** Thyroid controversy – stimulating antibodies. *J. Clin. Endocrinol. MeTab.* 1998. **83:** 3777–3785.

6 **Shimojo, N., Kohno, Y., Yamaguchi, K., Kikuoka, S., Hoshioka, A., Niimi, H., Hirai, A., Tamura, Y., Saito, Y., Kohn, L. D. and Tahara, K.,** Induction of Graves-like disease in mice by immunization with fibroblasts transfected with the thyrotropin receptor and a class II molecule. *Proc. Natl. Acad. Sci. USA* 1996. **93:** 11074–11079.

7 **Costagliola, S., Many, M. C., Denef, J. F., Pohlenz, J., Refetoff, S. and Vassart, G.,** Genetic immunization of outbred mice with thyrotropin receptor cDNA provides a model of Graves' disease. *J. Clin. Invest.* 2000. **105:** 803–811.

8 **Akamizu, T., Matsuda, F., Okuda, J., Li, H., Kanda, H., Watanabe, T., Honjo, T. and Mori, T.,** Molecular analysis of stimulatory anti-thyrotropin receptor antibodies (TSAbs) involved in

Graves' disease. Isolation and reconstruction of antibody genes, and production of monoclonal TSAbs. *J. Immunol.* 1996. **157:** 3148–3152.

9 **Li, H., Akamizu, T., Okuda, J., Sugawa, H., Matsuda, F., Tsubata, T. and Mori, T.,** Isolation of Epstein-Barr-virus-transformed lymphocytes producing IgG class monoclonal antibodies using a magnetic cell separator (MACS): preparation of thyroid-stimulating IgG antibodies from patients with Graves' disease. *Biochem. Biophys. Res. Commun.* 1995. **207:** 985–993.

10 **Akamizu, T., Moriyama, K., Miura, M., Saijo, M., Matsuda, F. and Nakao, K.,** Characterization of recombinant monoclonal antithyrotropin receptor antibodies (TSHRAbs) derived from lymphocytes of patients with Graves' disease: epitope and binding study of two stimulatory TSHRAbs. *Endocrinology* 1999. **140:** 1594–1601.

11 **Saijo, M., Akamizu, T., Moriyama, K., Miura, M., Hattori, Y. and Nakao, K.,** *In vivo* bioactivities of recombinant monoclonal thyroid stimulating antibodies derived from lymphocytes of patients with Graves' disease. *81th Annual Meeting of The Endocrine Society,* San Diego 1999, pp P1–P353.

12 **Silva, J. E.,** The multiple contributions of thyroid hormone to heat production. *J. Clin. Invest.* 2001. **108:** 35–37.

13 **Murakami, M., Tsubata, T., Okamoto, M., Shimizu, A., Kumagai, S., Imura, H. and Honjo, T.,** Antigen-induced apoptotic death of Ly-1 B cells responsible for autoimmune disease in transgenic mice. *Nature* 1992. **357:** 77–80.

14 **Okamoto, M., Murakami, M., Shimizu, A., Ozaki, S., Tsubata, T., Kumagai, S. and Honjo, T.,** A transgenic model of autoimmune hemolytic anemia. *J. Exp. Med.* 1992. **175:** 71–79.

15 **Hayakawa, K., Shinton, S. A., Asano, M. and Hardy, R. R.,** B-1 cell definition. *Curr. Top. Microbiol. Immunol.* 2000. **252:** 15–22.

16 **Hardy, R. R. and Hayakawa, K.,** B cell development pathways. *Annu. Rev. Immunol.* 2001. **19:** 595–621.

17 **Murakami, M., Tsubata, T., Shinkura, R., Nisitani, S., Okamoto, M., Yoshioka, H., Usui, T., Miyawaki, S. and Honjo, T.,** Oral administration of lipopolysaccharides activates B-1 cells in the peritoneal cavity and lamina propria of the gut and induces autoimmune symptoms in an autoantibody transgenic mouse. *J. Exp. Med.* 1994. **180:** 111–121.

18 **Sakiyama, T., Ikuta, K., Nisitani, S., Takatsu, K. and Honjo, T.,** Requirement of IL-5 for induction of autoimmune hemolytic anemia in anti-red blood cell autoantibody transgenic mice. *Int. Immunol.* 1999. **11:** 995–1000.

19 **Afeltra, A., Paggi, A., Ferri, G. M., Amoroso, A., Di Prima, M. A., Startari, S., Faralli, A. R. and Bonomo, L.,** $CD5^+$ B lymphocytes and $CD4^+$ $CD45RA^+$ T cells in Graves' disease. *Endocr. Res.* 1993. **19:** 73–85.

20 **Corrales, J. J., Orfao, A., Lopez, A., Ciudad, J. and Mories, M. T.,** Serial analysis of the effects of methimazole therapy on circulating B cell subsets in Graves' disease. *J. Endocrinol.* 1996. **151:** 231–240.

21 **Goodnow, C. C.,** B-cell tolerance. *Curr. Opin. Immunol.* 1992. **4:** 703–710.

22 **Santamaria, P.,** Effector lymphocytes in autoimmunity. *Curr. Opin. Immunol.* 2001. **13:** 663–669.

23 **Endo, T., Ohta, K., Haraguchi, K. and Onaya, T.,** Cloning and functional expression of a thyrotropin receptor cDNA from rat fat cells. *J. Biol. Chem.* 1995. **270:** 10833–10837.

24 **Rapoport, B., Chazenbalk, G. D., Jaume, J. C. and McLachlan, S. M.,** The thyrotropin (TSH) receptor: interaction with TSH and autoantibodies. *Endocr. Rev.* 1998. **19:** 673–716.

25 **Weetman, A. P., Yateman, M. E., Ealey, P. A., Black, C. M., Reimer, C. B., Williams, R. C., Jr., Shine, B. and Marshall, N. J.,** Thyroid-stimulating antibody activity between different immunoglobulin G subclasses. *J. Clin. Invest.* 1990. **86:** 723–727.

26 **Shin, E. K., Akamizu, T., Matsuda, F., Sugawa, H., Fujikura, J., Mori, T. and Honjo, T.,** Variable regions of Ig heavy chain genes encoding antithyrotropin receptor antibodies of patients with Graves' disease. *J. Immunol.* 1994. **152:** 1485–1492.

27 **Byun, C. H., Park, J. Y., Akamizu, T. and Chae, C. B.,** Identification of the peptides that inhibit the function of human monoclonal thyroid-stimulating antibodies from phage-displayed peptide library. *J. Clin. Endocrinol. MeTab.* 2001. **86:** 3311–3318.

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