

Analysis of Cellular Immunity in Patients with Graves' Disease

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ABSTRACT

Graves' disease has attracted considerable attention as an autoimmune disease. In this study, cellular immunity in patients with this disease was assessed. Specifically examined was the lymphocyte response to mitogens, Interleukin-2 (IL-2) production from peripheral blood mononuclear cells (PBMCs) and the percentage of lymphocyte subsets. No significant difference was observed in the lymphocyte response to phytohemagglutinin (PHA), concanavalin A (Con A) and pokeweed mitogen (PWM) between untreated patients with Graves' disease and healthy people. IL-2 production in untreated patients, however, was significantly greater than that of healthy people. While a significant decrease was observed in the percentage of CD8⁺ cells in untreated patients, no difference was found in the percentage of CD5⁺, CD4⁺ and HLA-DR⁺ cells between them and healthy people. It is thought that the enhancement of IL-2 production by PBMCs and the decrease in the percentage of CD8⁺ cells (cytotoxic/suppressor cell) are associated with abnormalities in the immune system.

Key words: Graves' disease, Interleukin-2 (IL-2), Lymphocyte subset, Mitogen

Two types of autoantibodies to the thyrotropin (TSH) receptor other than anti-microsome antibody and anti-thyroglobulin antibody are known to exist in thyroid disease sera¹⁸⁾. One is a thyroid-stimulating antibody (TsAb) which stimulates thyroid cells and enhances thyroid hormone production. The other is a TSH binding inhibitor antibody (TBIAb) which inhibits the binding of TSH to the TSH receptor on thyroid cells. Since the pathogenesis of Graves' disease appears to be associated particularly with TsAb, Graves' disease is generally regarded as an autoimmune disease.

Abnormality of cellular immunity has been observed in autoimmune diseases^{2,15,21)}. Many laboratories have reported on Graves' disease with little agreement in their findings^{4,5,12)}.

Interleukin-2 (IL-2) is a T-cell growth factor essential for normal immunological reaction. Decrease of IL-2 production by PBMCs has been reported for several autoimmune diseases²⁾, but the relationship between the decrease and the mechanism through which autoimmune diseases develop is not clear. In this report, we studied the lymphocyte response to various mitogens, IL-2 production from PBMCs, and the percentage of T cell subsets in patients with Graves' disease. Our aim was to examine the relationship between the changes in the immune system and development of the disease in the participating patients.

MATERIALS AND METHODS

SAMPLES: Blood samples were obtained from 53 patients with Graves' disease (19 males and 34 females, ages: 18-65 years, mean \pm S.D. = 40.0 \pm 10.6) and 51 normal subjects (20 males and 31 females, ages 20-61 years, mean \pm SD = 40.7 \pm 10.5) as shown in Table 1. These subjects did not have the immunological disorders such as autoimmune disease and hematological disease.

Cells: PBMCs were prepared from defibrinated venous blood by Ficoll-Hypaque density centrifugation as described previously¹⁾.

Lymphocyte culture: PBMCs were suspended in Eagle's minimum essential medium (Eagle's MEM) containing 5% heat inactivated pooled human serum, 2mM L-glutamine and 1% non-essential amino acid (Gibco) at cell concentration of 3.1×10^5 /ml. Two hundred μ l of cell suspensions were dispensed into each well of a U-bottomed microtest plate (Nunc), phytohemagglutinin (PHA: Wellcome), concanavalin A (Con A: Sigma Co.) or pokeweed mitogen (PWM: Gibco Co.) was added at a final concentration of 10 μ l/ml, 20 μ g/ml and 5 μ l/ml, respectively, and the cells were cultured in 5% CO₂ at 37°C for three days. At 18 hr prior to completion of culture, 0.5 μ Ci of ³H-thymidine (³H-TdR) (NEN, 5 Ci/mmol) was added. The cells were harvested on glassfiber filters and radioactivity was determined.

Table 1. Subjects

Disease	Therapy	No. of subjects			Age (yrs)		Concentration of	
		Male	Female	Total	Range	Mean \pm S.D.	T ₃ ^{a)} (ng/ml)	T ₄ ^{a)} (μ g/dl)
Normal	None	20	31	51	20-61	40.7 \pm 10.5	N.D.	N.D.
Graves'	None	4	8	12	23-65	40.9 \pm 11.9	5.96 \pm 3.13	21.76 \pm 6.49
Graves'	Drug alone ^{b)}	4	15	19	18-60	40.4 \pm 13.9	2.63 \pm 2.28	10.78 \pm 7.64
Graves'	Drug + RI ^{c)}	12	11	23	23-65	38.2 \pm 9.4	2.59 \pm 1.49	10.3 \pm 5.4

a) Normal range of T₃ is 1.3–2.2 ng/ml and that of T₄ is 5.3–10.9 μ g/dl.

b) Patients received anti-thyroid drugs such as thiamazole and propylthiuracil.

c) Patients received with radioisotope in addition to anti-thyroid drugs.

Detection of lymphocyte subsets: Lymphocyte subsets were detected by indirect immunofluorescence method using monoclonal antibody as described previously⁸⁾. The monoclonal antibodies employed were anti-Leu 3a (CD4), anti-Leu 1 (CD5), anti-Leu 2a (CD8), anti-HLA-DR and anti-Leu 7 (CD57) (Becton Dickinson Co.). As the second antibody, fluorescein isothiocyanate (FITC)-labeled affinity-purified goat anti-mouse IgG (Tago) was used. After staining, more than 200 PBMCs were counted with a fluorescence microscope, and the percentage of membrane fluorescence positive cells was calculated. The counting was conducted by one person without knowledge of the disease, age or sex of the subjects.

IL-2 production

One million lymphocytes from the subjects were suspended in 1 ml of test medium consisting of RPMI-1640 with 4 mM HEPES (hydroxyethyl-piperazine N'-ethane sulfonic acid), 100 U/ml of penicillin, 100 μ g/ml streptomycin, 1% L-glutamine, 1% PHA-M (DIFCO Co.) and 2% heat-inactivated fresh human pooled AB serum.

After incubation for 24 hours at 37°C in a 5% CO₂ incubator, the supernatant was harvested and stored at -80°C until assay. Culture supernatant was serially diluted two-fold in Click's medium and added to the wells of microplates in aliquots of 0.1 ml each. To each well, the IL-2-dependent cytotoxic T cell line (CTLL-2) was added at the rate of 4 \times 10³ cells/well and cultured at 37°C in a 5% CO₂ incubator for 24 hours. Four hours before harvesting, 0.4 μ Ci of ³H-TdR was added. Cultures were harvested onto glass fiber filter strips, and ³H-TdR incorporated by cells was determined.

Units of IL-2 production were determined by probit analysis¹⁰⁾. The maximum value of ³H-TdR incorporation (counts per minute, cpm) in CTLL-2 cells on serial log₂ dilution of standard IL-2 was designated as 100% and cpm of an IL-2 sample was converted into %. These values were plotted on the Y axis (probit axis) and the dilution multiples of the sample on the X axis (log₂ axis) to obtain the regression line. The value on the X axis at 50% activity of standard IL-2 was designated as 1 unit and the unit of the IL-2 sample was computed with the following equation. Unit = IL-2 sample dilution multiple at 50% activity of standard IL-2/standard IL-2 dilution multiple at 50% activity of standard IL-2.

Statistical analysis:

The Wilcoxon rank sum test was employed for statistical processing of the data.

RESULTS

Lymphocyte responses to mitogens (Table 2):

There was no significant difference in the lymphocyte response to PHA between untreated or treated patients with Graves' disease, and healthy people.

No significant difference in the lymphocyte response to Con A was observed between untreated patients and healthy people. In contrast, the lymphocyte response in patients treated with anti-thyroid drugs such as thiamazole or propylthiuracil and radioisotope was significantly lower (5,521 \pm 4,097 cpm) than that in healthy people (8,962 \pm 6,608 cpm) ($p < 0.05$).

Neither was there any significant difference in the lymphocyte response to PWM between untreated

Table 2. Lymphocyte responses to mitogens in patients with Graves' disease

Disease	Therapy	No. of cases	Lymphocyte response to		
			PHA (cpm)	Con A (cpm)	PWM (cpm)
Normal	None	44	39,841 \pm 33,429 ^{a)}	8,962 \pm 6,608	6,017 \pm 3,933
Graves'	None	12	32,844 \pm 23,343	7,164 \pm 4,425	5,749 \pm 1,998
Graves'	Drug alone ^{b)}	17	30,107 \pm 20,704	6,875 \pm 3,467	3,727 \pm 1,991**
Graves'	Drug + RI ^{c)}	20	36,752 \pm 23,521	5,521 \pm 4,097*	6,658 \pm 3,013

a) Mean S.D.

b) See Table 1

c) See Table 1

*,** indicates statistical significance (* $p < 0.05$, ** $p < 0.01$) between these groups and normal group.

Table 3. Lymphocyte subsets in patients with Graves' disease

Disease	Therapy	No. of cases	Lymphocyte subset				
			CD5 ⁺ (%)	CD4 ⁺ (%)	CD8 ⁺ (%)	CD4/CD8	HLA-DR ⁺ (%)
Normal	None	50	63.8 ± 8.8 ^{a)}	39.9 ± 9.6	23.6 ± 6.0	1.9 ± 1.0	14.8 ± 5.9
Graves'	None	12	61.4 ± 10.2	40.8 ± 7.7	19.8 ± 5.7*	2.2 ± 0.7*	13.6 ± 5.0
Graves'	Drug alone ^{b)}	18	62.7 ± 9.9	44.1 ± 9.8	22.4 ± 7.9	2.4 ± 1.5	11.7 ± 4.3*
Graves'	Drug + RI ^{c)}	23	66.4 ± 11.9	44.1 ± 9.6	22.9 ± 6.3	2.1 ± 0.7*	13.9 ± 6.0

a) Mean ± S.D.

b), c) See Table 1.

* p<0.05 by Wilcoxon rank sum test between these groups and normal group.

Table 4. IL-2 production in patients with Graves' disease

Disease	Therapy	No. of cases	IL-2 production (U/ml)
Normal	None	51	0.97 ± 0.54
Graves's	None	11	1.59 ± 0.72**
Graves'	Drug alone ^{b)}	18	1.36 ± 1.19
Graves'	Drug + RI ^{c)}	20	0.97 ± 0.47

a) Mean ± S.D.

b), c) See Table 1.

** indicates statistical significance (**p<0.01) between these groups and normal group.

ed patients and healthy persons. However, the lymphocyte response to PWM in patients who received only anti-thyroid drugs was $3,727 \pm 1,991$ cpm. This value was significantly lower than that in healthy persons ($6,017 \pm 3,933$ cpm) ($p < 0.05$).

Lymphocyte subsets (Table 3):

There was no significant difference in the percentage of CD5⁺ and CD4⁺ cells in PBMCs between the patients with Graves' disease and the normal subjects. The percentage of CD8⁺ cells in PBMCs in untreated patients ($19.8 \pm 5.7\%$) was significantly lower than that in healthy people ($23.6 \pm 6.0\%$) ($p < 0.05$). In contrast, no significant difference was observed in the percentage of CD8⁺ cells between treated patients and healthy people. The CD4/CD8 ratios were 2.2 ± 0.7 and 2.1 ± 0.7 respectively in untreated patients and patients treated with anti-thyroid drugs and radioisotope, both ratios being significantly higher than that in healthy people (1.9 ± 1.0) ($p < 0.05$). There was no significant difference in the percentage of HLA-DR⁺ cells in PBMCs between untreated patients and normal persons. However, the percentage of HLA-DR⁺

cells in PBMCs in the patients treated with anti-thyroid drugs was significantly lower than that in healthy people.

IL-2 production by PBMC (Table 4):

IL-2 production by PBMCs in untreated patients with Graves' disease was significantly greater (1.59 ± 0.72 U/ml) than that in healthy people (0.97 ± 0.54 U/ml) ($p < 0.01$). Little difference, however, was observed in IL-2 production between treated patients and healthy people.

Correlation between thyroid function and immunological parameters (Table 5):

The correlations between the level of triiodothyronine (T3) and thyroxine (T4) in the sera of Graves' disease patients and the lymphocyte response to mitogens, IL-2 production and the percentage of lymphocyte subsets were examined. The level of T4 showed a positive correlation with the percentage of HLA-DR positive cells, but no correlation with other immunological parameters. No correlation was observed between any of the these immunological parameters and the level of T3.

DISCUSSION

The lymphocyte response to mitogens in patients with Graves' disease was assessed. There was no significant difference in the lymphocyte response to mitogens between untreated patients with Graves' disease and healthy people. Many laboratories have conducted similar experiments, and have reported different results. Some have reported a decrease in the responsiveness of the patients and others have indicated no difference between the patients and healthy people^{5,12}. The present study showed that lymphocyte response to ConA and PWM decreased in patients who were treated with anti-thyroid drugs. The mechanism is unknown. However, there

Table 5. Correlation coefficient (r) between thyroid function and immunological parameters in all patients with Graves' disease

Thyroid hormones	Lymphocyte response to			Lymphocyte subsets						
	PHA	Con A	PWM	Leu 1	Leu 2a	Leu 3a	$\frac{\text{Leu 3a}}{\text{Leu 2a}}$	HLA-DR	Leu 7	IL-2 production
T ₃	-0.06	0.02	0.11	0.10	0.11	-0.06	-0.09	0.16	-0.15	0.06
T ₄	0.095	0.13	0.16	0.09	0.10	-0.04	-0.08	0.22*	-0.19	0.08

* Correlation between serum T₄ level and HLA-DR was statistically significant ($p < 0.05$)

is the possibility that activated suppressor-like T cells suppress the lymphocyte responses to mitogens, since it was reported that methimazole therapy induced the circulation of activated suppressor-like T cells¹⁷.

Further, the percentage of T cell subsets in patients with Graves' disease was assessed, and we observed a decrease in the percentage of CD8⁺ cells in untreated Graves' disease patients. Similar results were reported by Stridama, et al¹⁴. Volpe et al reported that, of the CD8⁺ cells, a decrease was confirmed in CD8⁺, CD11⁺ cells which are thought to be suppressor T cells⁹. It was pointed out that patients with Graves' disease might not be able to control autoantibody production due to the decrease of suppressor T cells.

Our assessment found no significant difference in the percentage of other cell populations (CD5, CD4, HLA-DR and CD57) between untreated patients and healthy people. However, reports from a multitude of laboratories lack a definitive trend: some have reported a decrease in the percentage of CD5⁺ cells¹¹ and CD3⁺ cells¹⁶, and a increase of HLA-DR⁺ cells^{3,19}.

Untreated patients had an acceleration of IL-2 production by PBMC. On the other hand, patients who had anti-thyroid drugs treatment showed no difference in the IL-2 production by PBMC from that of healthy people. In contrast to Graves' disease patients, the IL-2 production decreased in untreated patients with chronic thyroiditis (in unpublished observation). These data may suggest that there is some relationship between the states of thyroid function and immunological function. Our analysis, however, indicated no significant correlation between the concentration of thyroid hormone in the sera and IL-2 production from PBMCs. Weetman also reported that thyroxine did not affect IL-2 production *in vitro*²⁰, and this may suggest little effect of thyroid hormone on the immune system. It may be elicited from the finding on the decrease of CD8⁺ cells and the reports on the impairment of suppressor cell function in patients with Graves' disease¹³ that IL-2 production has been accelerated not by the direct effect of thyroid hormone but by the effect of immunological mechanism. There also is a possibility that IL-2 production in Graves' disease patients will be normalized by the treatment with radioisotope. This is reported to induce the decrease of helper inducer T cells⁶. Although few reports have observed IL-2 production in patients with Graves' disease, there are, in contrast with our results, reports that have indicated a decrease in IL-2 production in untreated active patients⁷. All the patients whom we examined were also active (serum levels of T₃ and T₄ were above normal range). As the presence of other different clinical status between the present study and this report is not known, the reason why this discrepancy arose is not known.

The relationship between various cytokines other than IL-2 and the development of Graves' disease has yet to be clarified and merits future investigation.

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