Ratio of Serum IgG\textsubscript{3} to Total IgG Concentration and Goiter Size Are Independent Factors in Intractability of Graves’ Disease

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Abstract. Peripheral immunoglobulin (Ig) G\textsubscript{3}-secreting cells and serum concentrations of interleukin (IL)-10, a class-switching factor to IgG\textsubscript{3}-secreting cells, increase in patients with intractable Graves’ disease (GD). However, they are not practical for laboratory tests. To find more stable and easily detectable markers of disease intractability or disease severity in patients with GD or Hashimoto’s disease (HD), we examined the serum concentration of IgG\textsubscript{3} in 58 euthyroid GD patients who had been undergoing antithyroid drug treatment for more than 5 years but still must continue drug treatment to maintain a euthyroid state (intractable GD), 26 GD patients who had maintained a euthyroid state for more than 2 years without any treatment (GD in remission), 20 untreated, thyrotoxic GD patients, 40 euthyroid HD patients treated with thyroxine (5 men and 35 women), 13 untreated, euthyroid HD patients, and 39 healthy volunteers. Serum concentrations of IgG\textsubscript{3} increased in euthyroid patients with intractable GD and in those with GD in remission, but serum concentrations of IgG were not altered. The ratio of serum concentrations of IgG\textsubscript{3} to total IgG (IgG\textsubscript{3}/IgG ratio) was higher in euthyroid patients with intractable GD than in those with GD in remission. Multiple logistic-regression analysis demonstrated that IgG\textsubscript{3}/IgG ratio and goiter size were independent factors in disease intractability of GD patients. These results suggest that IgG\textsubscript{3}/IgG ratio and goiter size may be used as independent markers associated with GD intractability.

Key words: Goiter, IgG\textsubscript{3}, Disease intractability, Graves’ disease

AUTOIMMUNE thyroid diseases such as Graves’ disease (GD) and Hashimoto’s disease (HD) are archetypal organ-specific autoimmune diseases [1–3]. The intractability of GD and the severity of HD vary between patients. Some patients with GD achieve remission through medical treatment. Most patients with HD maintain a lifetime euthyroid state without any medical treatment, whereas others become hypothyroid. Immune differences that underlie differences in the severity and intractability of these diseases remain unclear, and disease severity and intractability are difficult to predict. However, we have shown that particular lymphocyte subsets [4–7] and serum soluble CD8 [8] are related to the severity and intractability of these diseases. The levels of TSH receptor antibody (TRAb), IL-4, and IgE are also reported as the factors involved in the prediction of remission in GD [9, 10].

In addition, we recently reported that IgG\textsubscript{3}-secreting cells, detected by enzyme-linked immunospot (ELISpot) assay, are increased in the peripheral blood of patients with intractable GD, who had been undergoing antithyroid drug treatment for more than 5 years but still must continue drug treatment to maintain a euthyroid state [11] and that serum concentrations of IL-10, a factor involved in the switch of Ig-secreting cells to IgG\textsubscript{3}-secreting cells [12, 13], are also increased in these patients [14]. However, clinical use of ELISpot assay to detect IgG\textsubscript{3}-secreting cells is not practical because of technical complexity and variable results [11]. Most cytokines are produced and act locally; serum cytokines are unstable, and their con-
Serum concentrations of IL-10 are difficult to detect in most patients and healthy individuals [14]. Serum IgG subclasses, whose levels are determined by cytokine balance [12, 13, 16], are stable, and concentrations are detectable [17]. We hypothesized that serum concentrations of IgG3 may be used as a clinical indicator of the intractability of GD to antithyroid drug therapy as well as goiter size.

In this study, we analyzed serum concentrations of IgG3 and total IgG in patients with autoimmune thyroid diseases to determine the significance of serum IgG3 concentration.

**Patients and Methods**

**Patients and control subjects**

The study group comprised 104 patients with GD, 53 patients with HD (Table 1), and 39 healthy volunteers. Patients with GD were classified into three groups: 58 euthyroid patients who had been undergoing antithyroid drug treatment for more than 5 years but still must continue drug treatment to maintain a euthyroid state (intractable GD) (10 men and 48 women), 26 patients in remission who had maintained a euthyroid state and were negative for TRAb for more than 2 years without any treatment (GD in remission) (1 man and 25 women), and 20 untreated and thyrotoxic patients (3 men and 17 women) who were positive for TRAb. The antithyroid drug used for treatment was methimazole or propylthiouracil. HD was diagnosed by antithyroid microsomal antibody (McAb) and/or antithyroglobulin antibody (TgAb) positivity, a negative TRAb level, and the presence of diffuse goiter. Patients with HD were classified into two groups: 40 euthyroid patients treated with thyroxine because of hypothyroidism caused by severe destruction of thyroid follicles (severe HD) (5 men and 35 women), and 13 untreated, euthyroid patients (mild HD) (1 man and 12 women). The control group consisted of 39 healthy volunteers (2 men and 37 women) who were euthyroid and negative for thyroid autoantibodies. All patients, with the exception of thyrotoxic GD patients, showed normal serum concentrations of free thyroxine (FT4), free triiodothyronine (FT3), and TSH (Table 1). The mean age in each GD group and each HD group did not differ significantly from that of the control subjects (40.8 ± 11.9 years), and there was no sex difference among these groups. No statistical difference was detected between the mean ages of the three GD groups and those of the two HD groups. Informed consent for the study was obtained from all patients. Protocols were approved by our local Ethics Committee.

**ELISA of total IgG and IgG3**

Ninety-six-well microtiter plates (Nunc-Immuno Module MaxiSorp; Nunc, Roskilde, Denmark) were coated with 100 µl/well monoclonal anti-human IgG3 (mouse IgG1 isotype) (Cat. No. I7260; Sigma-Aldrich, St. Louis, MO) or anti-human IgG (mouse IgG2a isotype) (Cat. No. I7261). Ninety-six-well microtiter plates were coated with 100 µl/well monoclonal anti-human IgG3 (mouse IgG1 isotype) (Cat. No. I7260; Sigma-Aldrich, St. Louis, MO) or anti-human IgG (mouse IgG2a isotype) (Cat. No. I7261).

| Table 1. Clinical characteristics of patients with autoimmune thyroid diseases |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Group           | n               | Age (yr)        | Free T4 (ng/dl) | Free T3 (pg/ml) | TSH (µU/ml) | TRAb (%)       | McAb (2ⁿ × 10⁰) | TgAb (2ⁿ × 10⁰) |
| Graves’ disease |                 |                 |                 |                 |             |                 |                 |                 |
| Thyrotoxic      |                 |                 |                 |                 |             |                 |                 |                 |
| untreated       | 20              | 39.4 ± 16.3     | 4.52 ± 1.49     | 13.98 ± 7.57    | ND          | 41.1 ± 28.6     | 4.1 ± 3.7       | 1.3 ± 2.0       |
| Euthyroid       |                 |                 |                 |                 |             |                 |                 |                 |
| intractable     | 58              | 40.4 ± 14.3     | 1.12 ± 0.38     | 2.90 ± 0.53     | 1.77 ± 1.50 | 19.9 ± 26.9     | 5.0 ± 3.5       | 1.1 ± 2.1       |
| in remission    | 26              | 45.7 ± 13.0     | 1.27 ± 0.20     | 2.83 ± 0.40     | 1.94 ± 1.53 | -3.9 ± 4.7      | 4.1 ± 3.5       | 1.0 ± 2.0       |
| Hashimoto’s disease |             |                 |                 |                 |             |                 |                 |                 |
| Euthyroid       |                 |                 |                 |                 |             |                 |                 |                 |
| severe          | 40              | 53.7 ± 12.7     | 1.53 ± 0.49     | 2.86 ± 0.42     | 1.46 ± 1.48 | ND              | 4.7 ± 4.0       | 2.3 ± 4.0       |
| mild            | 13              | 47.0 ± 14.3     | 1.29 ± 0.25     | 2.81 ± 0.25     | 1.96 ± 1.16 | ND              | 2.7 ± 3.3       | 1.5 ± 2.0       |

Results are expressed as mean ± standard deviation (SD). ND, not determined; T4, thyroxine; T3, triiodothyronine; TSH, thyrotropin; TRAb, anti-thyrotropin receptor antibody; McAb, anti-thyroid microsomal antibody; TgAb, anti-thyroglobulin antibody
IgG3 type (Cat. No. I6760; Sigma-Aldrich), suspended at a concentration of 1.0 µg/dl (for IgG3) and 300.0 µg/dl (for IgG) in 0.05 M carbonate buffer (Na2CO3, NaHCO3, pH 9.6). After incubation overnight at 4°C, the wells were washed four times with phosphate-buffered saline (PBS, pH 7.2) containing 0.05% Tween 20 (PBST). The wells were then blocked for 60 min at room temperature with 200 µl PBST containing 0.1% bovine serum albumin (BSA; Sigma-Aldrich) (BSA-PBST). After four washes in PBST, 150 µl of serum sample (diluted 1 : 1,000 for IgG3 or 1 : 100,000 for IgG in 0.1% BSA-PBST) or serum standard (diluted 1 : 100–1 : 6,400 for IgG, or 1 : 10,000–1 : 640,000 for IgG) was added to each well. After a 180 min-incubation at room temperature, the wells were washed four times with PBST, and 100 µl horseradish peroxidase-conjugated goat anti-human IgG (γ-chain specific) F(ab')2 fragment (Cat. No. A2290; Sigma-Aldrich) was added to each well. After a 180 min-incubation at room temperature, the wells were washed four times with PBST, and 100 µl 1,2-phenylenediamine dihydrochloride solution (DAKO, Kyoto, Japan) was added to each well. After 30 min, the enzymatic reaction was terminated by adding 100 µl 1 M H2SO4 to each well, and absorbance was measured at 492 nm.

Assay of thyroid function and autoantibody levels

The serum concentration of free T4 (FT4) was measured with a radioimmunoassay kit (Eiken Chemical Co., Ltd., Tokyo, Japan). The normal range of serum FT4 is 1.0–1.6 ng/dl (12.9–20.6 pmol/L). Serum concentration of free T3 (FT3) was also measured with a radioimmunoassay kit (Japan Kodak Diagnostic Co., Ltd., Tokyo, Japan). The normal range of serum FT3 is 2.4–4.6 pg/ml (3.8–7.2 pmol/L). Serum TSH concentration was also measured with a radioimmunoassay kit (Daichi Radioisotope Laboratories Ltd., Tokyo, Japan). The normal range of serum TSH is 0.6–5.4 µU/ml. McAb and TgAb were measured with a particle agglutination kit (Fujirebio Inc., Tokyo, Japan). A reciprocal titer of ≥1 : 100 was considered positive. Serum TRAb was measured with a radioreceptor assay (Cosmic Corp., Tokyo, Japan); results were expressed as percent inhibition of binding of labeled TSH. The cut-off value is less than 10%.

Statistical analysis

Mann-Whitney U test was used to analyze differences in serum IgG and IgG3 concentrations between two groups of patients with GD of different levels of intractability or between two groups of patients with HD of different levels of severity, and the Kruskal-Wallis test and Dunn test were used for multiple comparisons among three or more groups. Spearman rank correlation test was used to analyze the correlation between serum IgG or IgG3 concentrations and serum titers of McAb or TgAb. Probability values <0.05 were considered significant. Pearson’s correlate coefficient was used to analyze the correlation between IgG/IgG3 ratios and the dose of antithyroid drug.

Odds ratios were used as a measure of association of disease intractability in euthyroid patients with intractable GD or GD in remission. Odds ratios were adjusted simultaneously for potentially confounding variables by multiple logistic-regression analysis. The variables considered were age (yrs), sex, IgG3/IgG ratio (%), goiter size (cm), FT4 (ng/dl), and TgAb levels (titer). Goiter size was measured by slide caliper and expressed as transverse diameter. In patients with non-palpable goiters, goiter size was expressed as 0 cm. We calculated 95% confidence intervals for all odds ratios. All probability values are two-tailed.

Results

Serum IgG and IgG3 concentrations

Serum IgG3 concentrations were significantly higher in euthyroid patients with intractable GD or GD in remission than in healthy subjects (P<0.001) (Fig. 1). However, serum IgG3 concentrations did not differ between untreated, thyrotoxic GD patients or euthyroid patients with severe HD or mild HD and healthy subjects. Serum IgG3 concentrations were higher in euthyroid patients with intractable GD than in thyrotoxic patients with GD (P<0.01). Serum concentrations of total IgG did not differ between groups of patients with GD or HD and healthy subjects.

IgG3/IgG ratios

IgG3/IgG ratios were significantly higher in euthyroid patients with intractable GD or GD in remission.
than in healthy subjects (P<0.001 and P<0.05, respectively) and were also higher in patients with intractable GD than in thyrotoxic GD patients (P<0.01). We used Mann-Whitney U test to analyze differences between two groups of euthyroid patients with GD. The IgG\textsubscript{3}/IgG ratio was higher in euthyroid patients with intractable GD than in those with GD in remission (P<0.05) (Fig. 2).

**Factors associated with GD intractability**

We evaluated factors associated with GD intractability by multiple logistic-regression analysis. Among the variables entered into the regression analysis, sex, IgG\textsubscript{3}/IgG ratio, and goiter size were identified as independent factors associated with GD intractability (Table 2).

**Sensitivity and specificity of prediction of disease intractability**

IgG\textsubscript{3}/IgG ratio and goiter size were independent factors associated with GD intractability. Therefore, values for patients with intractable GD or GD in remission were plotted two-dimensionally in Figure 3. With a cut-off value for IgG\textsubscript{3}/IgG ratio of >17.6%, 10 of 58 patients with intractable GD were positive (sensitivity: 17.2%), and 25 of 26 patients with GD in remission were negative (specificity: 96.3%). With a cut-off value for goiter size of >6.0 cm, 11 of 58 patients with intractable GD were positive (sensitivity: 19.0%), and 26 of 26 patients with GD in remission were negative (specificity: 100%). When we combined the factors, 22 of 58 patients with intractable GD were positive for at least one of the two factors (sensitivity: 37.9%), and 25 of 26 patients with GD in remission were negative for both factors (specificity: 96.3%).

![Figure 1. Serum concentrations of (a) IgG and (b) IgG\textsubscript{3} in patients with Graves’ or Hashimoto’s disease and control subjects. Significant differences were detected by Kruskal-Wallis test and Dunn test for multiple comparisons.](image)
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Effect of thyrotoxicosis on IgG$\text{\textsubscript{3}}$/IgG ratio

The IgG$\text{\textsubscript{3}}$/IgG ratio was inversely correlated with the serum concentration of FT4 in all patients with GD, including euthyroid and thyrotoxic patients ($n = 104$, $r = -0.26$, $P<0.01$). An inverse correlation was also observed between IgG$\text{\textsubscript{3}}$/IgG ratio and FT3 level in all GD patients ($n = 104$, $r = -0.21$, $P<0.05$). There were no significant correlations between IgG$\text{\textsubscript{3}}$/IgG ratio and TSH, McAb, and TgAb levels in these GD patients. There was no correlation between IgG$\text{\textsubscript{3}}$/IgG ratio and the dose of antithyroid drugs in euthyroid patients with intractable GD (Fig. 4). A serial study of IgG$\text{\textsubscript{3}}$/IgG ratios of six GD patients during treatment with antithyroid drugs and two untreated GD patients with transient thyrotoxicosis showed that IgG$\text{\textsubscript{3}}$/IgG ratio increased significantly from $13.6 \pm 9.4\%$ to $18.1 \pm 14.5\%$ as the thyroid hormone levels changed from high to normal (Fig. 5). Transient thyrotoxicosis due to aggravation of GD was diagnosed by the clinical course of transient increase of TRAb without the following increase of TSH.

### Table 2. Independent factors associated with intractability of Graves’ disease

<table>
<thead>
<tr>
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<th>Odds ratio</th>
<th>95% confidence interval</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>3.17$^a$</td>
<td>1.20–14.9</td>
<td>0.049</td>
</tr>
<tr>
<td>IgG$\text{\textsubscript{3}}$/IgG ratio</td>
<td>1.13$^b$</td>
<td>1.02–1.28</td>
<td>0.034</td>
</tr>
<tr>
<td>Goiter size</td>
<td>1.41$^c$</td>
<td>1.10–1.86</td>
<td>0.010</td>
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$^a$ Odds ratio for male/female.

$^b$ Odds ratio for 1% increase in IgG$\text{\textsubscript{3}}$/IgG ratio.

$^c$ Odds ratio for 1 cm increase in goiter size.

Fig. 2. Ratios of serum concentrations of IgG$\text{\textsubscript{3}}$ to IgG (IgG$\text{\textsubscript{3}}$/IgG ratio) in patients with Graves’ or Hashimoto’s disease and control subjects. Significant differences were detected by Kruskal-Wallis test and Dunn test for multiple comparisons and by *Mann-Whitney U test.

Fig. 3. Two-dimensional analysis of IgG$\text{\textsubscript{3}}$/IgG ratios and goiter size in patients with Graves’ disease. Dotted lines show cut-off values for prediction of disease intractability.
Discussion

In this study, we found that the IgG\textsubscript{3}/IgG ratio was higher in euthyroid patients with intractable GD than in those with GD in remission and that there was no difference in this ratio between euthyroid patients with severe HD and those with mild HD (Fig. 2). These findings are consistent with our previous findings that the numbers of IgG\textsubscript{3}-secreting cells are increased in euthyroid patients with intractable GD and that there is no difference in the numbers of these cells between euthyroid patients with severe HD and those with mild HD [11]. We suggested that, in patients with intractable GD, type 2 helper T (Th2) cells [16] may be relatively predominant and produce IL-10 [14], a Th2-type cytokine [16], followed by increased numbers of IgG\textsubscript{3}-secreting cells [11] and increased serum concentration of IgG\textsubscript{3}, as shown in this study. Compared to measuring the number of IgG\textsubscript{3}-secreting cells by ELISPOT assay, it is easy to measure serum concentrations of IgG and IgG\textsubscript{3} by ELISA. Concentrations of IgG and IgG\textsubscript{3} in serum were much higher and more stable [17] than were concentrations of cytokines such as IL-10 [15]. We supposed that these differences in basal IgG\textsubscript{3}/IgG ratio may be defined by some genetic factor. Therefore, serum IgG\textsubscript{3}/IgG ratio may be used as a clinical marker associated with GD intractability.

It has been reported that the thyroid-stimulating antibody is IgG\textsubscript{1} [18]. Our previous data that serum IL-10 increases in patients with intractable GD [14] appears

![Fig. 4. Correlation between dose of antithyroid drug and IgG\textsubscript{3}/IgG ratio in patients with Graves’ disease during treatment.](image)

**Thyroid function**

![Fig. 5. Serial changes in serum concentration of free T4 (a) and IgG\textsubscript{3}/IgG ratio (b) in patients with Graves’ disease during treatment with antithyroid drugs (solid lines) and in untreated patients with transient thyrotoxicosis (dotted lines); thyrotoxic and euthyroid conditions are shown.](image)
to be consistent with this report because IL-10 is also a switching factor for IgG [12, 13]. In support of our result, IgG1 and IgG3 subclasses were predominant in serum antibodies to the acetylcholine receptor in patients with myasthenia gravis [19]. However, we did not find any differences in the numbers of IgG1-secreting cells between patients with intractable GD and those with GD in remission [11]. It may be difficult to detect the changes in the numbers of IgG1-secreting cells which were induced by a particular cytokine because there are many more cytokines which are switching factors for IgG1 than those for IgG3.

It is well known that goiter size is also associated with GD intractability [20–24]. In the present study, we confirmed this and clarified that the specificity of goiter size in the detection of patients with intractable GD was very high (cut-off value: >6.0 cm) although its sensitivity was very low. Furthermore, by multiple logistic-regression analysis, we found that serum IgG3/IgG ratio and goiter size are independent factors in GD intractability (Table 2). These findings suggest that serum IgG3/IgG ratio and goiter size may represent different factors of GD intractability, and that their combined analysis may be useful in the detection of patients with intractable GD under treatment with antithyroid drugs.

In GD patients, serum IgG3/IgG ratios were higher in those that were euthyroid than in those with thyrotoxicosis (Fig. 5). IgG3/IgG ratios were inversely correlated with serum concentrations of thyroid hormones. We found similar changes in IgG3/IgG ratios in GD patients with untreated, transient thyrotoxicosis (dotted lines in Fig. 3). There was also no significant involvement of the dose of antithyroid drug with IgG3/IgG ratios (Fig. 4). Therefore, it is unlikely that treatment with antithyroid drugs affect these ratios. Thyroid hormones may reduce the IgG3/IgG ratio, and it is important to measure the IgG3/IgG ratio in the euthyroid state.

In conclusion, serum IgG3/IgG ratio and goiter size may be used as independent indicators of GD intractability in euthyroid patients with GD under treatment with antithyroid drugs.

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References


