Existence and immunological significance of circulating Ia⁺T cells in autoimmune thyroid diseases

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Abstract. We investigated the percentage of circulating HLA-DR antigen positive (Ia antigen positive: Ia⁺) T cells and the additive proliferation by non-specific mitogens and thyroid-specific antigens by means of a cytotoxicity test in autoimmune thyroid diseases. Furthermore, we studied the stimulative function of circulating Ia⁺T cells in autologous mixed lymphocyte reactions. %Ia⁺T cells were significantly increased in patients with autoimmune thyroid diseases compared with those in normal controls. They were additionally increased by the stimulation of TSH-receptor or thyroid-microsome in patients with Graves' disease, and by the stimulation of thyroglobulin and thyroid-microsome in patients with Hashimoto's thyroiditis. As to the cellular immune function, circulating Ia⁺T cells stimulated Ia⁺T cells in autologous MLR in patients with autoimmune thyroid diseases. These data suggest that some of the T cells are already activated in vivo, that the activation of T cells may be by thyroid-specific antigens, and that these activated (Ia⁺) T cells may be able sequentially to induce the activation of inactivated (Ia⁺) T cells in autoimmune thyroid diseases.

HLA-DR antigens are known not to be expressed on normal circulating T cells, but it has recently been clarified that HLA-DR antigens are expressed on immunologically activated T cells, as well as monocytes, macrophages, and B cells (Fu et al. 1978). It is apparent from previous studies that HLA-DR antigen positive T cells (Ia antigen positive T cells: Ia⁺T cells) frequently appear in the circulation in systemic autoimmune diseases, for instance rheumatoid arthritis and systemic lupus erythematosus (Yu et al. 1980; Jackson et al. 1982; Fukui et al. 1984). Furthermore, in such diseases, it is reported that the variations in circulating Ia⁺T cells are related to the disease activity (Matsumoto & Wakazono 1982).

In this paper, we investigated the existence of circulating Ia⁺T cells in vivo and the changes of %Ia⁺T cells by stimulation of thyroid-specific antigens in vitro in patients with autoimmune thyroid diseases. In addition, we studied the stimulatory function of circulating Ia⁺T cells in autologous mixed lymphocyte reaction to examine the cellular immune function of circulating Ia⁺T cells.

Materials and Methods

Subjects
The subjects of this investigation include 16 untreated patients with Graves' disease (13 females and 3 males), 22 patients in euthyroid state under a regimen of methimazole (17 females and 5 males), 25 patients with Hashimoto's thyroiditis (22 females and 3 males), and 10 normal controls (9 females and 1 male).

Preparation of peripheral blood mononuclear cells
Mononuclear cells were isolated from heparinized venous blood by centrifugation on a lymphocyte separation medium (Litton Bionetics, Kensington, MD) at 400 × g for 30 min, washed twice with phosphate buffered saline (PBS) and resuspended at 5 × 10⁹ cells/l in RPMI 1640 culture medium (GIBCO) with 10% FCS (GIBCO).
Preparation of T cells
To isolate T cell subsets, nylon wool purification was carried out by using mononuclear cells as mentioned above. To identify the cell populations passed through the column, cells were passed through a Spectrum 3 flow cytofluorimeter (Ortho Diagnostic Systems) using an argon ion laser at 488 nm excitation wavelength and counted at each fluorescence intensity. The cells were monitored by markers of T cells (OKT3, Ortho Diagnostic Systems), B cells (Bl, Coulter Co), and monocytes (OKM5, Ortho Diagnostic Systems). In this population, OKT3+ cells were over 86%, Bl+ cells were less than 3%, and OKM5+ cells were less than 2%.

Thyroid-specific antigens
Thyroid-microsome, thyroglobulin and TSH-receptor preparations were used as thyroid-specific antigens. The thyroid-microsome and thyroglobulin antigens (Fuji Rebio. Co, Tokyo, Japan) were the same used as antigens in Japanese commercial kits for measurement of antithyroglobulin (MCHA: microsome haemagglutination) and anti-thyroglobulin (TGHA: thyroglobulin haemagglutination) antibody titres. The thyroid-microsome antigen was prepared from homogenized surgical specimens of human thyroids by differential centrifugation technique. Briefly, a crude fraction of thyroid-microsome was first obtained as follows: the homogenized human thyroids were centrifuged in 0.15 mol/l KCl at 1100 × g for 30 min and the supernatant thus acquired was centrifuged in 0.15 mol/l KCl at 10000 × g for 15 min. Finally, the supernatant thus gained was centrifuged in 0.15 mol/l KCl at 78000 × g for 90 min and the precipitation was used as crude fraction of thyroid-microsome. Thereafter, this crude fraction of thyroid-microsome was centrifuged in 0.15 mol/l NaCl at 78000 × g for 90 min and the supernatant thus obtained was centrifuged in the same way. The last precipitation acquired from these procedures was suspended in 0.15 mol/l NaCl and used as thyroid-microsome antigen. The thyroglobulin antigen was fractioned from the last supernatant gained from the procedures mentioned above by 36-46% ammonium sulphate and suspended in PBS. The TSH-receptor was obtained from Smith’s kit which is used for measurement of TSH-receptor antibody activity. This TSH-receptor is prepared from porcine thyroid membranes by differential centrifugation and extracted using 1.0% Lubrol 12 A9 in 50 mmol/l NaCl, 10 mmol/l Tris-HCl, pH 7.5, and 3 mmol/l NaN3 (Smith & Hall 1981).

Proliferative assays
Each subject’s peripheral blood mononuclear cells were suspended at 5 × 10⁵ cells/l in RPMI 1640 (GIBCO) with 10% FCS (GIBCO) whereafter non-specific mitogens, namely Con A (Sigma Chemical Co, St. Louis, MO) or PWM (GIBCO) were added to the cell suspensions at final concentrations of 20 mg/l, together with thyroid-specific antigens such as TSH-receptor, thyroglobulin at final concentrations of 50 mg/l, or thyroid-microsome at a final dilution of 1:200. Thereafter, the incubation was performed for 4 days at 37°C in a moist atmosphere of 5% CO₂.

Detection of Ia+T cells
To detect Ia+T cells, cytotoxicity test was performed in duplicate and %cytotoxicity was regarded as %Ia+T cells. In 96-well round-bottom microtitre plates (Sanko Junyaku Co, Tokyo, Japan), 40 µl (2 × 10⁵) cells of T cells (nylon wool purified cells) were incubated with 40 µl of 10 × murine monoclonal antibody against framework determinant of Ia antigen (Leu-HLA-DR antibody: Becton-Dickinson Co, Oxnard, CA) and 40 µl of 10 × rabbit complement (Cedarlane Laboratories Ltd, Ontario, Canada) for 1 h at 37°C in a moist atmosphere of 5% CO₂. Then, %cytotoxicity was calculated as follows by trypan blue (GIBCO) dye exclusion of dead cells:

% cytotoxicity = (1 - 
 survived cell counts 
 total cell counts 
) × 100.

Per cent cytotoxicity was calculated on the day of blood-gathering for the detection of %Ia+T cells and on the 4th day of proliferative assays mentioned above. Per cent Ia+T cells was expressed as mean %cytotoxicity of duplicate cytotoxicity tests.

Removal of Ia+T cells
To obtain a Ia negative (Ia⁻) T cell rich population, unfractioned (T cells prepared as mentioned in Preparation of T cells) 1 × 10⁷ T cells were incubated with 100 µl of 10 × murine monoclonal antibody against framework determinant of Ia antigen (Leu-HLA-DR antibody: Becton-Dickinson Co, Oxnard, CA) and 100 µl of 10 × rabbit complement (Cedarlane Laboratories Ltd, Ontario, Canada) for 1 h at 37°C in a moist atmosphere of 5% CO₂, washed 3 times in PBS and resuspended at 5 × 10⁶ cells/l in RPMI 1640 (GIBCO) with 10% FCS (GIBCO).

Autologous mixed lymphocyte reaction (MLR)
 Cultures were performed in triplicate in 96-well flat-bottom microtitre plates (Falcon 3072, Becton-Dickinson, Oxnard, CA) that contained 5 × 10⁴ stimulator cells (unfractioned T cells or Ia⁻ T cell rich population) and 5 × 10⁴ responder cells (Ia⁺ T cell rich population) originated from the same subject in 200 µl of medium RPMI 1640 (GIBCO) with 10% FCS (GIBCO). The stimulator cells were pretreated with 50 mg/l of mitomycin C (Sigma Chemical Co, St. Louis, MO) at 37°C for 60 min, washed 3 times with PBS and resuspended in RPMI 1640 (GIBCO) with 10% FCS (GIBCO) before culture. Following 4 days of culture at 37°C in a moist atmosphere of 5% CO₂, 0.25 µCi/well of [³H]thymidine (Amersham, Japan Co, Tokyo, Japan) was added and...
incubation was continued for an additional 24 h. Cells were then harvested on glass fibre filters with an automatic cell harvester (Shimadzu Seisakusho Ltd, Kyoto, Japan) and the radioactivity of each sample was determined by liquid scintillation counter.

Results

**Circulating %IA+T cells in autoimmune thyroid diseases**

Fig. 1 shows the circulating %IA+T cells in autoimmune thyroid diseases. Circulating %IA+T cells was significantly increased in patients in both hyperthyroid state (17.3 ± 9.4: mean ± SD) and euthyroid state (8.9 ± 8.6) of Graves' disease, and also in patients with Hashimoto's thyroiditis (15.2 ± 10.9) as compared with that of normal controls (2.0 ± 2.7). %IA+T cells in the patients in euthyroid state of Graves' disease was lower than in the patients in hyperthyroid state, and also it was markedly decreased in euthyroid patients with negative TRAb (5.2 ± 6.3: not shown in the figure). Circulating %IA+T cells in subacute thyroiditis and nodular goitre did not exceed the normal range (not shown).

**Changes of %IA+T cells by proliferative assays**

Changes of %IA+T cells by stimulation of non-specific mitogens such as Con A or PWM were examined. Compared with the values without stimulation (9.6 ± 6.7: mean ± SD), %IA+T cells was significantly increased by the stimulation of Con A (30.9 ± 11.7) and PWM (32.3 ± 12.3) in Graves' disease, and it was significantly increased too by the stimulation of Con A (35.4 ± 11.8) and PWM (34.4 ± 8.0) in Hashimoto's thyroiditis compared with cells without stimulation (11.5 ± 7.4). In addition, %IA+T cells was increased by the stimulation of Con A (30.3 ± 7.8) and PWM (33.6 ± 4.4) compared with cells without stimulation (2.2 ± 2.5) in normal controls, and the degree of increased %IA+T cells did not differ between normal controls and autoimmune thyroid diseases, namely Graves' disease or Hashimoto's thyroiditis (Fig. 2).

Fig. 3 shows changes of %IA+T cells by stimulation of thyroid-specific antigens. Although the changes were less pronounced than those by stimulation of non-specific mitogens, %IA+T cells was increased by the stimulation of TSH-receptor (20.4 ± 6.4) and thyroid-microsome (27.7 ± 7.8) as compared with cells without stimulation (10.2 ±

![Fig. 1.](image_url)

Circulating %IA+T cells in autoimmune thyroid diseases. *: P < 0.01; #: P < 0.05. Student's t-test.

0: TRAb (-), •: TRAb (+). TRAb: TSH receptor antibody titre assayed by Smith's kit.
5.4: mean ± SD), but not by the stimulation of thyroglobulin (11.4 ± 3.7) in Graves' disease. On the other hand, in Hashimoto's thyroiditis, %Ia+T cells was increased by the stimulation of thyroglobulin (11.9 ± 8.1) and thyroid-microsome (21.9 ± 13.6), but not by the stimulation of TSH-receptor (10.4 ± 9.0) compared with cells without stimulation (9.8 ± 7.0). There were no increase in %Ia+T cells in normal controls by the stimulation of TSH-receptor (2.3 ± 2.5) or thyroglobulin (2.3 ± 2.5) or thyroid-microsome (2.4 ± 2.4) compared with cells without stimulation (2.2 ± 2.3).

Relationships between %Ia+T cells and the other immunological indices

We furthermore examined the relationships between %Ia+T cells and the other immunological indices. As shown in Fig. 4, %Ia+T cells correlated significantly with TRAb (TSH-receptor antibody titre assayed by Smith's kit), but there were no significant correlations between %Ia+T cells and anti-microsomal or anti-thyroglobulin antibody titres in Graves' disease. In Hashimoto's thyroiditis, no significant correlations were demonstrated between %Ia+T cells and titres of any autoantibodies.

Although not shown in the figure, it was demonstrated that circulating %Ia+T cells is related to the activity of the disease in Graves' disease from the longitudinal observation of the clinical course in each case.

Stimulative function of circulating Ia+T cells in autologous MLR

We compared the stimulatory function of unfractioned T cells with Ia+T cell rich population in

**Table 1.**

<table>
<thead>
<tr>
<th>Source of responder Ia+T cells</th>
<th>Stimulator cells</th>
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<tbody>
<tr>
<td></td>
<td>Ia+T cell rich population (cpm)</td>
</tr>
<tr>
<td>Graves' disease</td>
<td></td>
</tr>
<tr>
<td>P 1</td>
<td>397 ± 122</td>
</tr>
<tr>
<td>P 2</td>
<td>1126 ± 35</td>
</tr>
<tr>
<td>P 3</td>
<td>785 ± 162</td>
</tr>
<tr>
<td>P 4</td>
<td>2042 ± 314</td>
</tr>
<tr>
<td>Hashimoto's thyroiditis</td>
<td></td>
</tr>
<tr>
<td>P 1</td>
<td>1801 ± 238</td>
</tr>
<tr>
<td>P 2</td>
<td>919 ± 141</td>
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<tr>
<td>P 3</td>
<td>679 ± 83</td>
</tr>
<tr>
<td>P 4</td>
<td>1195 ± 74</td>
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<tr>
<td>Normal controls</td>
<td></td>
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<tr>
<td>C 1</td>
<td>456 ± 5</td>
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<tr>
<td>C 2</td>
<td>1310 ± 469</td>
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<tr>
<td>C 3</td>
<td>1004 ± 294</td>
</tr>
<tr>
<td>C 4</td>
<td>1339 ± 68</td>
</tr>
</tbody>
</table>

Compared with the value of 'Ia+T cell rich population' in the same patient.
* P < 0.01 and ** P < 0.05 (Student's t-test).

The culture of autologous MLR was conducted using 5 × 10⁴ stimulator cells (unfractioned T cells or a Ia+T cell rich population) pre-treated with mitomycin C and a equal number of responder Ia+T cells in microtitre plates for 5 days at 37°C in a moist atmosphere of 5% CO₂. Results of triplicate cultures are expressed as mean counts per min ± SD.
Changes of %Ia+T cells by stimulation of thyroid-specific antigens. * Symbol denotes significant difference ($P < 0.05$) when compared with no stimulant (paired $t$-test). NS indicates not significant difference when compared with no stimulant (paired $t$-test).

Fig. 3.

Fig. 4.

$T =$ Hyperthyroid state
$O =$ Euthyroid state

$\text{TRAb} : TSH$ receptor antibody assayed by Smith's kit

Relationship between % Ia+T cells and TRAb in Graves' disease.
autologous MLR. As shown in Table 1, unfractioned T cells can stimulate a Ia\(^{-}\)T cell rich population in autologous MLR in autoimmune thyroid diseases, on the other hand, a Ia\(^{-}\)T cell rich population did not have the stimulatory function. In normal controls, even unfractioned T cells did not have the capacity to stimulate a Ia\(^{-}\)T cell rich population.

Discussion

It is well known that in autoimmune thyroid diseases, the major site of immunological reactions is in the thyroid gland itself, and several investigators have tried to elucidate the pathogenesis of autoimmune thyroid diseases by use of peripheral blood mononuclear cells. Since 1982 it has been reported that circulating Ia\(^{+}\)T cells are increased in Graves' disease and Hashimoto's thyroiditis (Canonica et al. 1982, 1983; Jackson et al. 1982; Bonnyns et al. 1983). Jackson et al. (1984) reported that all 33 patients with Graves' disease in hyperthyroid state had increased values of %Ia\(^{+}\)T cells, whereas 8 patients with Graves' disease in euthyroid state after thyroid ablation had normal values of %Ia\(^{+}\)T cells. Furthermore, Ludgate et al. (1984) demonstrated that in Graves' disease the proportion of activated (Ia\(^{+}\)) T cells was significantly raised before treatment with carbimazole as compared with normal. It fell to normal by the end of a six-month course with the same anti-thyroid drug, but in patients who relapsed after treatment, there was a rise in the proportion of activated (Ia\(^{+}\)) T cells, which again returned to normal when treatment was re-installed. These studies and our present data demonstrated that T cells in peripheral blood have been previously activated in vivo in autoimmune thyroid diseases.

However, the effect of thyroid-specific antigens on the level of %Ia\(^{+}\)T cells has never been reported. In this investigation, we could demonstrate that, whereas there was no increase in %Ia\(^{+}\)T cells by the stimulation of thyroid-specific stimulant in normal controls, %Ia\(^{+}\)T cells were increased by the stimulation of TSH-receptor and thyroid-microsome in Graves' disease, and also it was increased by the stimulation of thyroglobulin and thyroid-microsome in Hashimoto's thyroiditis. In this investigation, we used the thyroid-microsome and thyroglobulin antigens and TSH-receptor as three different thyroid-specific antigens. Although we understand that these preparations were not in completely purified form and intrinsic limitations to exist for the final interpretation of our results, we are tempted to speculate that these data may be the result of specific effects for each different thyroid-specific antigen. In fact, there was no correlation between the titres of the three corresponding autoantibodies detected in 16 patients (i.e. anti-microsomal, anti-thyroglobulin, and TSH-receptor antibody, respectively assayed by commercial kits). In conclusion, we would like to suggest the hypothesis that at least, in vitro, thyroid-specific antigens may activate T cells, but the antigen involved in the stimulation of T cells seems to be different in Graves' disease and Hashimoto's thyroiditis. We also believe that this positive trend could be even more significant if mononuclear cells are prepared directly from the thyroid gland instead of those obtained from peripheral blood.

As to the cellular immune function of circulating Ia\(^{+}\)T cells, little is known about how these cells operate in autoimmune thyroid diseases. However, there are reports describing that activated Ia\(^{+}\)T cells acquire the ability to stimulate autologous and allogenic T cells in mixed lymphocyte reactions, and T cell proliferation in vitro by soluble antigens has been obtained by using non-specific mitogens and induced Ia\(^{+}\)T cells from normal subjects (Engleman et al. 1980; Mingari et al. 1981; Russo et al. 1982).

As far as autoimmune thyroid diseases are concerned, only Canonica et al. (1984) have reported that Ia antigens are involved in thyroglobulin-induced peripheral blood T cell proliferation in Hashimoto's thyroiditis. As to with activated (Ia\(^{+}\)) T cells within thyroid glands, it has been reported that these cells can stimulate inactive (Ia\(^{-}\)) T cells within thyroid glands and peripheral blood (Matsunaga et al. 1986). In the present paper, we demonstrated that circulating unfractioned T cells are capable of stimulating autologous Ia\(^{-}\)T cells, but a Ia\(^{-}\)T cell enriched population does not have the same stimulatory function in autoimmune thyroid diseases. This stimulatory function is supposed to be due to the circulating activated (Ia\(^{+}\)) T cells which are rich in unfractioned T cells in patients with autoimmune thyroid diseases, since circulating unfractioned T cells do not have the capacity to stimulate Ia\(^{-}\)T...
cells in normal controls. In conclusion, it is suggested that these Ia\(^+\)T cells previously activated with thyroid-specific antigens can induce the sequential activation of inactive (Ia\(^-\)) T cells in autoimmune thyroid diseases.

Although we could suggest that various thyroid-specific antigens activated circulating T cells in Graves' disease and Hashimoto's thyroiditis, and that the activated (Ia\(^+\)) T cells may induce the sequential activation of inactive (Ia\(^-\)) T cells, the precise role of circulating Ia\(^+\)T cells remains to be further elucidated in autoimmune thyroid diseases.

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References


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