Methimazole therapy in Graves’ disease influences the abnormal expression of CD69 (early activation antigen) on T cells

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Abstract

At present, the in vivo response of T, B and natural killer (NK) cells to antithyroid drug therapy remains largely unknown. In the present study, we have prospectively analyzed the in vivo effects of methimazole treatment on a large number of circulating T and NK cell subsets, some of them expressing cell surface activation antigens involved in the very early phase of the immune response, in a group of 17 hyperthyroid, untreated patients with Graves’ disease (GD). As one of the first events during T cell activation is the expression of interleukin (IL) receptors, we also studied the binding of IL-2 and IL-6 to T cells. Patients with Graves’ disease were sequentially studied at diagnosis/before treatment (day 0) and 7, 14, 30, 90 and 180 days after methimazole therapy. The results were compared with both a group of 19 age- and sex-matched control volunteers and a group of 20 untreated/euthyroid patients with Graves’ disease in long-term remission. The combination of flow cytometry and three-color immunofluorescence revealed a clear (P<0·01) decrease in the percentage of NK cells before and during the whole course of therapy with respect to both controls and patients with Graves’ disease who were in long-term remission. Before therapy, a marked increase (P<0·001) in the ratio of B to NK cells was also observed; thereafter, a slight decrease in this ratio was observed, although normal values were detected only in patients in long-term remission. Expression of the CD69 early activation antigen in the hyperthyroid untreated patients with Graves’ disease was clearly increased (P<0·01) with respect to both controls and patients with Graves’ disease who were in long-term remission. This abnormal CD69 expression was found to be significantly reduced (P<0·001) by methimazole therapy, and this represents a new effect of the drug. Expression of the low-affinity receptor for IL-2 (CD25) – another early T cell activation marker – was not altered in Graves’ disease, but the binding of IL-2 and IL-6 to T cells exhibited a progressive and parallel increase during the first 30 days of therapy, decreasing thereafter. Our results show that methimazole therapy downregulates the abnormally high expression of the CD69 early activation antigen on T cells, being less effective on inducing changes in other T cell activation markers and in NK cells.

Introduction

Antithyroid drugs have a well-defined biochemical effect, reducing thyroid hormone biosynthesis by inhibiting the action of thyroid peroxidase (Engler et al. 1983), and also an immunosuppressive effect, as shown by the decrease in the lymphocytic infiltration of the gland and in the serum concentrations of thyroid autoantibodies (Ratanachaiyavong & McGregor 1985). However, the mechanisms involved in this immunological action in Graves’ disease remain largely unknown (Soliman et al. 1995).

It has been suggested that the immunological effects of antithyroid drugs could be due both to an effect on thyrocyte–immunocyte signaling and to a reduction in thyroid hormone production (Volpé 1994). Intercellular signaling could be induced both through soluble factors (cytokines) or by cell–to–cell contact mediated by surface molecules (Smith 1988). Regarding the former, it has been shown that antithyroid drugs prevent the release of potent phlogistic mediators such as prostaglandin E2, interleukin (IL)-1α and IL-6 from thyrocytes (Weetman et al. 1992). Moreover, antithyroid drugs also restore the reduced IL-2 production by peripheral blood lymphocytes in Graves’ disease to normal levels (Eisenstein et al. 1994). With respect to cell–to–cell interactions, some work has been carried out on B cell subsets (Corrales et al. 1996a, b), but other types of lymphocytes such as T and NK cell subpopulations have also been reported to be intimately related to the key autoimmune process in Graves’ disease (Volpé 1994). In this sense, it has been shown that, in Graves’ disease, methimazole gradually reduces the
number of circulating activated (HLA-DR+) T helper/inducer (T_{H1}) cells and increases the number of circulating activated (HLA-DR+) T suppressor/cytotoxic (T_{S/C}) cells (Tötterman et al. 1987). In addition to these slow effects, induced over weeks to months of therapy, a rapid increase in the number of activated/suppressor T cells and T_{S/C} cells, and a decrease in the number of activated T helper and NK cells have been observed after only a few days of treatment (Karlsson & Tötterman 1988).

In order to gain further insight into the immunological effects of antithyroid drugs on cell signaling, in the present study we prospectively analyzed the serial effects of methimazole on a broad range of circulating T and NK cell subsets (Table 1), many of which have so far not been studied in Graves’ disease. Moreover, in order to investigate the influence of antithyroid drugs on early phases of T cell activation, we analyzed the expression of rapidly induced antigens such as CD69 and CD25, which become detectable in the cell membrane within a few hours after T cell activation has been triggered in response to antigenic stimuli (Hara et al. 1986, Testi et al. 1989a, López-Cabrera et al. 1993). Because one of the first events during T cell activation is the expression of IL receptors, we also studied the binding of IL-2 and IL-6 to peripheral blood T cells.

### Patients and Methods

#### Patients

Thirty-seven patients with Graves’ disease enrolled consecutively in our outpatient clinic and 19 age- and sex-matched healthy volunteers were studied prospectively. Seventeen of the patients with Graves’ disease were untreated, newly diagnosed cases in the active/hyperthyroid phase of the disease, according to clinical, hormonal and immunological criteria. There were 14 women and three men, ranging in age from 20 to 59 years (mean ± s.d. 35 ± 11 years). These patients were studied at diagnosis/before treatment (day 0), and at 7, 14, 30, 90 and 180 days after starting methimazole therapy. Patients were initially treated with 30–40 mg of methimazole per day. After 1 month, the dose of methimazole was reduced by half and maintained at that level throughout the study (6 months). After another 1 month, 50 µg levothyroxine was added to the drug regime of ten patients, to maintain euthyroidism. The remaining 20 patients with Graves’ disease were in long-term clinical and biochemical remission (average of 3–1 years); they comprised 16 women and four men, aged 19–63 years (mean ± s.d. 37 ± 13 years). Remission was obtained by previous treatment with methimazole or iodine-131 therapy; at the time of the study, these patients were taking no medication.

Diagnosis of Graves’ disease was based on the presence of symptoms and signs of thyroid hyperfunction, diffuse goiter, occasional ophthalmopathy, diffuse and homogeneous uptake in thyroid gammagraphy with technetium-99m, increased serum concentrations of thyroxine, tri-iodothyronine and antibodies to the thyrotropin (TSH) receptor (TSH-binding inhibitory immunoglobulin, TBII).

### Thyroid hormones and autoantibodies

Thyroid hormones and TSH concentrations were measured in the serum of all patients by RIA and by an amplified immunoradiometric assay (for TSH), according to previously described techniques (Corrales et al. 1991, Mories et al. 1991). For the determination of serum TBII concentrations, the ability of antibodies to block the binding of 125I-labelled TSH to porcine thyroid membranes was measured by a radioreceptor assay using...
a commercial kit (TRAK-Assay, Henning, Berlin, Germany). For the determination of antithyroid peroxidase (TPO) antibodies in serum, a solid-phase RIA was used (Henning, Germany). Serum concentrations of antithyroglobulin (TG) antibodies were measured by a two-step immunoradiometric assay (Cis Bio Int., France). In all cases, thyroid autoantibodies were studied in peripheral blood samples obtained simultaneously with respect to both those used for TBII and thyroid hormone serum concentration measurements and for the study of peripheral blood lymphocyte subsets.

**Immunological studies**

In all cases, EDTA-anticoagulated peripheral blood samples were obtained by venipuncture at 0800–0900 h. Analysis of the different lymphoid subsets was performed on erythrocyte-lysed whole blood using a stain-and-lyse protocol (Corrales et al. 1996b). Briefly, 100–200 µl peripheral blood containing 0·5–1 × 10⁶ nucleated cells were incubated with the appropriate combinations of monoclonal antibodies for 10 min at room temperature, then 2 ml fluorescence-activated cell sorting (FACS) lysing solution (Becton/Dickinson, San Jose, CA, USA) were added and the system incubated for a further 10 min in the dark. Cells were then centrifuged and washed once in 2 ml/tube PBS. The number of total T, B, NK, T-CD4+, T-CD8⁺, T-CD8⁻, T-CD4+/CD8⁺, T-CD4⁻/CD8⁻, NK-CD8⁻, NK-CD8⁺ cells were obtained using the Lymphogram™ (Landerdiagnostics, Madrid, Spain) reagent (US patent No. 5538855). In addition, the following monoclonal antibody combinations (FITC/PE/PerCP or PE/Cy5) were used: CD5/CD23/CD19; CD69/CD25/CD3. Of these reagents, CD5, CD23, CD69, CD25 and CD3 were purchased from Becton/Dickinson and CD19 from Caltag Laboratories (San Francisco, USA).

In order to measure the binding of both IL-2 and IL-6 to T cells, IL-2 and IL-6 conjugated with phycoerythrin (British Bio-Technology Products Ltd, Abingdon, Oxon, UK) were used. Briefly, between 100 and 200 µl EDTA-anticoagulated peripheral blood containing 0·5–1 × 10⁶ leukocytes were stained for the CD3 (Leu 4-PerCP, Becton/Dickinson) antigen after incubating the cells with this monoclonal antibody for 10 min in the dark at room temperature. Erythrocytes were then lysed by incubating the cells with 2 ml FACs lysing solution for another 10 min in the dark. Then, cells from lysed whole blood were washed twice (2 × 500 g, 5 min) in commercial RDF-1 washing buffer (British Bio-Technology Products Ltd) and resuspended in RDF-1 washing buffer at a concentration of 4 × 10⁶/ml. Immediately after washing, 10 µl phycoerythrin-conjugated IL-2, 10 µl phycoerythrin-conjugated IL-6 and 10 µl phycoerythrin-conjugated Ig-negative control were added to 25 µl washed cell suspension in three separate siliconized glass tubes. Cells were incubated for 60 min at 4°C. After this incubation, unbound cytokines were removed by washing twice in 2 ml/tube of RDF-1 washing buffer (2 × 500 g, 5 min) and resuspended in 0·5 ml PBS (pH=7·6). As a negative control, cells incubated with purified IL-2 and IL-6 before the IL-2–PE and IL-6–PE staining were also analyzed.

Measurements of both T and NK lymphoid subsets identified by monoclonal antibodies and the ability of T cells to bind IL-2 and IL-6 were performed on a FACScan flow cytometer (Becton/Dickinson) equipped with a 15 mW argon ion laser tuned at 488 nm. The instrument was adjusted using CD4–FITC, CD8–PE and CD8–PE/Cy5 stained lymphocytes and the LYSYS II software program (Becton/Dickinson). Lysys II and PAINT-A-GATE PRO software programs were used for data acquisition and data analysis, respectively. A minimum of 15 000 events were acquired for the Lymphogram™ monoclonal antibody combination. For the remaining tube combinations, data were obtained in a two-step procedure performing acquisition in the second step through a live gate established on SSC/CD3 or SSC/CD5 for T cells; a minimum of 5 000 events were stored in each live gate.

**Statistical analysis**

In order to analyze whether the differences between groups were statistically significant, Student’s unpaired and paired t-tests were used. For comparison of more than two groups, the inferential study was performed using analysis of variance (ANOVA) and, in cases where ANOVA was statistically significant, the cause of significance was explored with the Fisher LSD test.

**Results**

Table 2 shows the mean (± s.d.) serum concentrations of thyroid hormones, TSH and thyroid antibodies in patients with Graves’ disease before (day 0), during the course of treatment and in the remission phase. During methimazole therapy, thyroid hormone serum concentrations progressively declined (P<0·0001), reaching values similar (P>0·05) to those found in patients with Graves’ disease who were in long-term remission after 90 days of therapy. As from day 30 of treatment, TBII serum concentrations were not significantly different from those found in patients with Graves’ disease who were in long-term remission. The anti-TPO Ab serum concentrations never attained normal values, being increased even in the patients with Graves’ disease who were in long-term remission. The absolute numbers and the percentages of the peripheral blood T lymphocyte subsets analyzed in the
Table 2: Serum thyroid hormone and thyroid antibody concentrations in Graves’ disease patients before (day 0), after treatment with methimazole (MMI), and in long-term remission. Data are expressed as mean ± SD.

<table>
<thead>
<tr>
<th>Patients</th>
<th>TT₄  (nm)</th>
<th>FT₄  (pm)</th>
<th>TT₃  (nm)</th>
<th>FT₃  (pm)</th>
<th>TSH  (mIU/ml)</th>
<th>TBII (U/l)</th>
<th>TPOAb (U/ml)</th>
<th>TGAb  (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated (day 0) (n=17)</td>
<td>289 ± 44**</td>
<td>69 ± 29**</td>
<td>6·6 ± 1·7**</td>
<td>207 ± 6·6**</td>
<td>0·08 ± 0·02</td>
<td>48 ± 57*</td>
<td>2693 ± 3111</td>
<td>213 ± 423</td>
</tr>
<tr>
<td>Treated MMI (n=17)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 7</td>
<td>212 ± 55††**</td>
<td>49 ± 14††**</td>
<td>3·7 ± 1††**</td>
<td>9·9 ± 3·5††**</td>
<td>0·09 ± 0·05</td>
<td>57 ± 73*</td>
<td>2600 ± 3229</td>
<td>165 ± 343</td>
</tr>
<tr>
<td>Day 14</td>
<td>193 ± 74††**</td>
<td>38 ± 23††**</td>
<td>3·6 ± 1·6††**</td>
<td>10·1 ± 5·6††**</td>
<td>0·09 ± 0·05</td>
<td>63 ± 76*</td>
<td>2776 ± 3356</td>
<td>207 ± 417</td>
</tr>
<tr>
<td>Day 30</td>
<td>146 ± 79††**</td>
<td>26 ± 17††**</td>
<td>2·7 ± 1·1††**</td>
<td>7·6 ± 3·5††**</td>
<td>0·27 ± 0·5</td>
<td>45 ± 33</td>
<td>1192 ± 2412</td>
<td>249 ± 464</td>
</tr>
<tr>
<td>Day 90</td>
<td>123 ± 91††</td>
<td>22 ± 23††**</td>
<td>2·5 ± 1·8††</td>
<td>6·6 ± 6·1††</td>
<td>10 ± 31</td>
<td>40 ± 39</td>
<td>1363 ± 1574</td>
<td>284 ± 656</td>
</tr>
<tr>
<td>Day 180</td>
<td>92 ± 40††</td>
<td>14 ± 7††</td>
<td>16 ± 0·4††</td>
<td>43 ± 10††</td>
<td>6·7 ± 8</td>
<td>19 ± 15</td>
<td>1897 ± 2827</td>
<td>31 ± 38</td>
</tr>
<tr>
<td>Long-term remission (n=20)</td>
<td>103 ± 30</td>
<td>17 ± 4</td>
<td>17 ± 0·4</td>
<td>44 ± 1·2</td>
<td>5·7 ± 11</td>
<td>8 ± 13</td>
<td>3362 ± 3628</td>
<td>680 ± 1258</td>
</tr>
</tbody>
</table>

TT₄, Total thyroxine (normal range 51–141 nm); FT₄, free thyroxine (normal range 10·4 ± 23·4 pm); TT₃, total tri-iodothyronine (normal range 1·2–2·8 nm); FT₃, free tri-iodothyronine (normal range 2·3–6·9 pm); TSH, thyrotropin (normal range 0·15–5·0 mIU/ml); TBII, TSH binding inhibitory immunoglobulin (normal range <15 U/l); TPOAb, thyroid peroxidase antibody (normal range <100 U/ml); TGAb, thyroglobulin antibody (normal range <50 U/ml). †P<0·01, with respect to hyperthyroid untreated patients with Graves’ disease (day 0); *P<0·05, **P<0·01, both with respect to patients with Graves’ disease who were in remission.
Long-term remission patients with Graves’ disease and those receiving treatment with methimazole, compared with the controls, when absolute numbers were taken into account. However, the percentage of NK cells was significantly reduced (P<0.01) before and during the whole course of therapy compared with both the control group and the long-term remission patients. The decrease in the percentage of total NK cells was attributable to the decrease of the CD8+ NK cell subpopulation (P<0.01), as the number of CD8− NK cells remained unchanged. When the B/NK ratio was followed longitudinally over the period of methimazole therapy (Fig. 1), a marked increase before therapy (day 0) was observed, with respect both to control (P<0.001) and to long-term remission patients (P<0.001). Later on, the B/NK cell ratio slightly decreased during the 6-month period of treatment, normal values (P>0.05 with respect to controls) being attained only in patients with Graves’ disease who were in long-term remission.

Figure 2 shows the percentage of CD69+ T cells and IL-2+ T cells binding to exogenous IL-2 and IL-6 respectively. *P<0.05, **P<0.01, ***P<0.001 compared with controls; †P<0.05, ††P<0.01, †††P<0.001 compared with remission.

different groups of patients with Graves’ disease are presented in Table 3. Both untreated hyperthyroid patients with Graves’ disease and those receiving treatment with methimazole had absolute and relative counts of total T and CD8+ T cell subsets similar to those observed both in controls and in patients with Graves’ disease who were in long-term remission. There was a significant (P<0.01) increase in the absolute number of CD4+ T cells found at days 30, 90 and 180 of treatment. When compared both with control volunteers and patients with Graves’ disease who were in long-term remission, hyperthyroid untreated patients displayed a significant increase (P<0.01) in T cells expressing the CD69 activation-associated marker, which remained high only at the beginning of methimazole therapy. In contrast, the number of T cells bearing the low-affinity IL-2 receptor (CD25+), and the absolute and relative numbers of T cells binding to exogenous IL-2 (IL-2+ T) and IL-6 (IL-6+ T), did not show any significant difference between patients with Graves’ disease (before and during treatment) and both control subjects and long-term remission patients. The only exception was a greater expression of IL-2+ and IL-6+ T cells observed at day 30 of treatment (P<0.05). No significant differences between the different groups of patients with Graves’ disease and controls were observed for the CD5+ T, CD8hi+ T, CD8bi+ T, CD4+/CD8+ T and CD4−/CD8− T cell subsets (results not shown).

Regarding the overall distribution of peripheral blood NK cells (Table 4), no significant differences were observed between untreated hyperthyroid patients with Graves’ disease and those receiving treatment with methimazole, compared with the controls, when absolute numbers were taken into account. However, the percentage of NK cells was significantly reduced (P<0.01) before and during the whole course of therapy compared with both the control group and the long-term remission patients. The decrease in the percentage of total NK cells was attributable to the decrease of the CD8+ NK cell subpopulation (P<0.01), as the number of CD8− NK cells remained unchanged. When the B/NK ratio was followed longitudinally over the period of methimazole therapy (Fig. 1), a marked increase before therapy (day 0) was observed, with respect both to control (P<0.001) and to long-term remission patients (P<0.001). Later on, the B/NK cell ratio slightly decreased during the 6-month period of treatment, normal values (P>0.05 with respect to controls) being attained only in patients with Graves’ disease who were in long-term remission.
The possible influence of the serum concentrations of both free T\textsubscript{3} and free T\textsubscript{4} on the circulating T lymphocyte subsets was analyzed in hyperthyroid untreated patients with Graves’ disease (day 0). Consistent correlations were found only for the number of T cells binding to IL-2 (IL-2+ T cells) and the concentration of CD4+/CD8+ T cells (Table 5).

**Discussion**

In our serial study, we did not observe any abnormalities in hyperthyroid untreated patients with Graves’ disease in the distribution of peripheral blood total T cells (CD3+) and their CD4+, CD8+, CD4+/CD8+, CD4−/CD8− T cell subsets. These results confirm, and expand, recent reports in which no changes in circulating total T cells, T\textsubscript{H/A} (CD4+) or T\textsubscript{S/C} (CD8+) were observed in hyperthyroid untreated patients with Graves’ disease (Iwatani et al. 1992, Walfish & Tseng 1992). Of the remaining T cell subsets analyzed, only the CD4+/CD8+ T cell subpopulation has previously been assessed, by Iwatani et al. (1992), who, like us, did not find an abnormal distribution of this subset in hyperthyroid patients with Graves’ disease. In addition, in the present study we did not observe any abnormalities in the distribution of peripheral blood total T cells and their subsets.

### Table 4 Absolute (per 10\textsuperscript{6}/l) and relative (%, in bold) number of peripheral blood NK cell subsets in untreated, methimazole (MMI)-treated and long-term remission patients with Graves’ disease, and in healthy controls. Data are expressed as mean ± S.D.

<table>
<thead>
<tr>
<th>Patients</th>
<th>Total NK (CD56+/CD3−)</th>
<th>NKCD8+</th>
<th>NKCD8−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated (n=17)</td>
<td>272 ± 381</td>
<td>108 ± 139</td>
<td>164 ± 245</td>
</tr>
<tr>
<td>Day 0</td>
<td>8·5 ± 7·3†</td>
<td>3·5 ± 2·8†</td>
<td>5·1 ± 4·8</td>
</tr>
<tr>
<td>Treated MMI (n=17)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 7</td>
<td>205 ± 121</td>
<td>87 ± 52</td>
<td>118 ± 77</td>
</tr>
<tr>
<td>Day 14</td>
<td>207 ± 134</td>
<td>88 ± 54</td>
<td>120 ± 84</td>
</tr>
<tr>
<td>Day 30</td>
<td>312 ± 369</td>
<td>164 ± 227</td>
<td>148 ± 167</td>
</tr>
<tr>
<td>Day 90</td>
<td>207 ± 134</td>
<td>88 ± 54</td>
<td>120 ± 84</td>
</tr>
<tr>
<td>Day 180</td>
<td>314 ± 259</td>
<td>148 ± 120</td>
<td>171 ± 143</td>
</tr>
<tr>
<td>Long-term remission (n=20)</td>
<td>299 ± 174</td>
<td>76 ± 32</td>
<td>172 ± 147</td>
</tr>
<tr>
<td>Controls (n=19)</td>
<td>324 ± 157</td>
<td>90 ± 49</td>
<td>168 ± 96</td>
</tr>
</tbody>
</table>

*P<0·01 compared with controls; †P<0·01 compared with remission.

**Figure 1** Peripheral blood B/NK lymphocyte ratio in patients with Graves’ disease followed longitudinally during methimazole therapy ● and in patients with Graves’ disease who were in long-term remission ○. Normal values are presented in the cross-hatched area. *P<0·001 compared with patients in long-term remission. Ratio established on the basis of the absolute number of cells.
not find a significant effect of methimazole therapy on most of the circulating T cell subsets analyzed. These results are in agreement with and further expand previous observations obtained with other antithyroid drugs (Chan & Walfish 1986, Iwatani et al. 1992, Walfish & Tseng 1992).

In contrast to total T cells, TH/I cells and TS/C cells (mostly inactive), marked abnormalities have been observed both in untreated patients with Graves’ disease (Ludgate et al. 1984, Chan & Walfish 1986, Tötterman et al. 1987, Walfish & Tseng 1992) and in patients treated with antithyroid drugs (Ludgate et al. 1984, Tötterman et al. 1987, Karlsson & Tötterman 1988, Walfish & Tseng 1992) regarding the distribution of activated TH/I and TS/C cells. To assess T cell activation in these studies, the expression of HLA-DR was analyzed. HLA-DR expression is a relatively late event during T cell activation, therefore in order to obtain both a more dynamic and earlier information about the effects of antithyroid drugs on T cell activation, we analyzed the expression of both CD69 and CD25 cell surface antigens in addition to the binding of IL-2 and IL-6 to T cells. CD69, also termed early activation antigen (Hara et al. 1986), is a cell surface protein that is not expressed by resting lymphocytes but is quickly induced (within 2 h) upon T cell activation, its expression being downregulated 8 h later (Hara et al. 1986, Testi et al. 1989a). This molecule is the first inducible cell surface antigen detectable during lymphoid activation, thus providing an ideal tool with which to monitor the very early events occurring after T cell activation has been triggered (Testi et al. 1989a). Reactivity for CD69 is followed by an increase in the expression of the low-affinity receptor for IL-2 (CD25, TAC, α-chain) (Nakamura et al. 1989, Testi et al. 1989b), which appears at the surface of T cells several hours later (Hara et al. 1986). Other activation markers, such as the HLA-DR,
are expressed at a later stage during the activation process (Hara et al. 1986). Binding of IL-2 and IL-6 to T cells, considered to be important steps in the immune response (Smith 1988, Kishimoto et al. 1992), requires the expression of their corresponding high-affinity receptors (Smith 1988, Kishimoto et al. 1992) and, together with CD25, could be considered as signs of both rapid and maintained T cell activation markers (Abbas et al. 1994). CD69+ T cells were markedly increased in hyperthyroid untreated patients with Graves’ disease, both in absolute and in relative numbers. Interestingly, methimazole therapy induced a rapid normalization of the number of peripheral blood CD69+ T cells. To the best of our knowledge, this is the first time that abnormalities in the expression of this early activation antigen on T cells have been described in Graves’ disease. Such an abnormally high expression is shown here to be corrected by methimazole. According to the functional role of CD69, which acts as a transmembrane signaling receptor in the very early phases of cell activation (López-Cabrera et al. 1993), the signal delivered by CD69 induces T cell proliferation in a CD25-dependent way (Nakamura et al. 1989, Testi et al. 1989b). Its decreased expression after methimazole therapy could represent one of the first measurable immunological effects of the drug detectable at the cellular level; as its expression is only transient during the early phases of T cell activation, measurement of CD69 expression could be a useful tool with which to monitor the immunological effects of methimazole therapy. The design of the present study did not permit us to distinguish between a direct effect of methimazole on CD69 expression or an effect mediated through its action on thyroid hormone synthesis; however, CD69+ T cells reached normal values at times of the follow-up when hyperthyroidism was still present (days 14 and 30 of treatment). Moreover, previous reports analyzing the distribution of peripheral blood lymphocytes in patients with non-toxic multinodular goiter and in patients with toxic multinodular goiter did not show differences in the distribution of peripheral blood activated T cells between both groups of patients (Corrales et al. 1994).

Despite the absence of changes in the distribution of circulating T cells expressing the low-affinity IL-2 receptor (CD25), as mentioned above, IL-2 and IL-6 binding to T cells was progressively increased during the first 30 days of methimazole therapy. This apparent discrepancy between the expression of CD25 and the binding of IL-2 can be explained by the fact that IL-2 binds to the high-affinity receptor (Smith 1988), a complex composed of α, β and γ chains (Taniguchi & Minami 1993), and therefore expression of the CD25 molecule may be quantitatively unrelated to IL-2 binding. The direct relationship of IL-2 binding to the serum concentrations of thyroid hormones extends previous observations – without functional meaning (Weetman 1994) – in which a correlation between the circulating concentrations of thyroid hormones and the serum concentrations of soluble IL-2 receptors was found (Koukkou et al. 1991). Taken together, these results suggest that hyperthyroidism is able to affect the mechanisms of immunoregulation, as previously indicated by others (Volpé 1994).

The relative numbers of circulating NK cells and the CD8+ NK cell subset were decreased in our hyperthyroid patients with Graves’ disease both at diagnosis and during methimazole therapy, thus confirming previous reports on untreated (Iwatani et al. 1984) and antithyroid drug-treated patients (Karlsson & Tötterman 1988). The discrepant results reported by other groups in untreated patients (Wang et al. 1988) are probably related to the different methods used for detecting and enumerating NK cells. A decrease in the number of NK cells has also been detected in the thyroid gland of untreated patients with Graves’ disease (Iwatani et al. 1993). Our findings support the notion that, in the hyperthyroid state, an immunosuppressive action on NK cells may exist (Wang et al. 1988). This decrease in the percentage of NK cells could be related to a Th2 pattern of cytokine secretion, which would also be responsible for the increased numbers of B cells previously described for the same patient group (Corrales et al. 1996b). The suppression of NK cells could be related to the increased numbers of B cells, as it has been shown that some NK cells may produce lysis of activated B cells (Nabel et al. 1982, Brieva et al. 1984). An inverse relationship between B and NK cells within the thyroid has recently been described (Aust et al. 1996); an increased B/NK cell ratio was found in hyperthyroid untreated patients with Graves’ disease. Interestingly, antithyroid drug therapy induced a slight decrease in the B/NK cell ratio, which remained high throughout the period of therapy, and returned to normal values only in patients who were in long-term remission.

One may certainly question whether the changes in activated T cells observed in the peripheral blood reflect those occurring within the thyroid gland. In previous studies analyzing both circulating and intrathyroidal activated (HLA-DR+) T lymphocytes from untreated patients with Graves’ disease, a marked increase in this cell population was observed in the gland as compared with peripheral blood (Tötterman et al. 1987, Eguchi et al. 1989, Walfish & Tseng 1992, Iwatani et al. 1993, Aust et al. 1996). In contrast, no differences were found between either source of lymphocyte as regards either the percentage of activated CD4+/HLA-DR+ cells within activated total T cells or the corresponding percentage of CD8+/HLA-DR+ cells in untreated patients with Graves’ disease (Walfish & Tseng 1992). Although no significant differences were found between intrathyroidal and peripheral blood samples for both the percentage of activated CD4+/HLA-DR+ and activated CD8+/HLA-DR+ cells within activated total T cells, their concentrations (and ratio) were significantly greater in untreated patients with Graves’ disease than in normal individuals.
patients with Graves’ disease, thus confirming previous thyroidlymphocytes bearing the IL-2 receptor (CD25) in lymphocytes, could not find an increase in the number of the immunophenotype of peripheral blood and thyroid in peripheral blood, markedly increased in comparison patients with Graves’ disease undergoing long-term treatment, although in these patients the proportion of Ta1+ T cells in the thyroid tissue was, while similar to that in peripheral blood, markedly increased in comparison with that of normal subjects. Aust et al. (1996), comparing the immunophenotype of peripheral blood and thyroid lymphocytes, could not find an increase in the number of thyroid lymphocytes bearing the IL-2 receptor (CD25) in patients with Graves’ disease, thus confirming previous reports from McIntosh et al. (1993). These results suggest that, although some, but not all, of the changes in the pattern of expression of T cell activation markers may appear more clearly in the thyroid gland, the same abnormalities become detectable in peripheral blood. At present, to the best of our knowledge, no studies are available comparing the expression of CD69+ on T cells in untreated patients with Graves’ disease and normal individuals.

Despite this possible limitation, in our study, the investigation of immunocompetent cells in peripheral blood from patients with Graves’ disease is not devoid of significance. First, upon activation through antigenic stimulation, T cells proliferate and recirculate. Secondly, growing evidence indicates that Graves’ disease, rather than being an organ-specific condition, is a multisystemic inflammatory disease involving many tissues (Wall 1995). Therefore, studies limited to the thyroid gland may provide a partial picture of the changes occurring in other tissues, including the peripheral blood. In addition, at present, clear evidence exists that a significant number of lymphocytes producing antibodies to TSH receptor are present in peripheral blood (Fan et al. 1994). Finally, in order to induce the pathogenic events in Graves’ disease, the proliferation of intrathyroidal lymphoid populations is insufficient by itself, and must eventually be completed by emigration of mononuclear cells from the peripheral blood to the gland, in a process in which the patterns of expression of cell adhesion molecules may be particularly relevant (Weetman & McGregor 1994).

In summary, in the present study we have shown that in untreated hyperthyroid patients with Graves’ disease there is an increase in the peripheral blood ratio of B/NK cells that slightly decreases during methimazole therapy, normal values being attained in patients in long-term remission. Methimazole exerts an inhibitory effect on the increased expression of the CD69 early activation antigen observed on circulating T cells, while no major changes are detected regarding the expression of the CD25 activation-associated antigen. The mechanisms underlying this effect, and the clinical value of these parameters in the monitoring of methimazole therapy in patients with Graves’ disease, remain to be confirmed in larger series of patients.

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