Serial analysis of the effects of methimazole therapy on circulating B cell subsets in Graves' disease

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Abstract

The immunosuppressive effects of antithyroid drug therapy are well recognized; however, the cellular mechanisms underlying their action remain largely unknown. In the present paper we have prospectively analyzed the in vivo effects of methimazole treatment on a large number of circulating B cell subsets, involved in the effector phase of the immune response, in a group of 18 hyperthyroid patients with Graves' disease (GD). The patients were sequentially studied before (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 healthy controls and a group of 20 untreated/euthyroid GD patients in long-term remission. The combination of flow cytometry and three colour immunofluorescence revealed a clear increase (P<0.001) in the numbers of circulating total B cells (CD19+) due to a significant increase (P<0.001) in the CD5+, FMC7+, CD5+/FMC7+ and CD23+ B cell subsets in hyperthyroid GD patients with respect to both healthy individuals and to GD patients in long-term remission. The absolute numbers of all these B cell subsets analyzed before treatment, although abnormal, were not statistically different from those observed during the whole period of therapy. When comparing the percentages of these B cell subsets during treatment, significant changes (P<0.001) were only observed in the proportion of CD5+, CD5+/FMC7+ and CD5− B cells at the end of the follow-up period with respect to those found both before and during the first month of therapy. Whereas CD5+ and CD5+/FMC7 + B cells decreased (P<0.001) after 3 months of therapy, CD5− B cells showed a significant increase (P<0.001) at the end of therapy. It is remarkable that the percentage of CD5+, CD5+/FMC7+, CD5− and CD23+ B cell subsets were abnormal during the whole period of treatment and that they never reached normal values. These results show that, in vivo, GD patients treated with methimazole exhibited an abnormal but rather stable pattern of B cell distribution, similar to that present in hyperthyroid untreated GD patients, except for the CD5+ and CD5− B cell populations. Our findings suggest that in vivo methimazole therapy would not directly have an important influence on circulating B cell subsets.

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Introduction

Antithyroid drug therapy inhibits thyroid hormone biosynthesis and induces a variable remission rate in patients with Graves' disease (GD) (Utiger 1995). Thyrostatic drugs also have an immunosuppressive effect, as shown both by the decrease in the lymphocytic infiltration of the gland and in the serum levels of thyroid autoantibodies (Ratanachaiyavong & McGregor 1985); however, the cellular mechanisms involved in this immunological action remain unknown.

Graves' disease, as other autoimmune conditions, is the pathological consequence of an autoimmune response. This reaction is initiated by the activation of T cells by an MHC II/self-antigen complex and a co-stimulatory signal that enables full activation of T cells (Harlan et al. 1995). T cell activation is followed by the secretion of soluble regulatory peptides (interleukins) and by the expression of cell surface antigens and cell adhesion molecules that allow the homing of immune cells onto their target organs as well as signalling between T and B cells (Rose 1989, Weetman 1991, Pozzilli et al. 1994, Schattner 1994). Afterwards, the so-called effector phase of the immune response (Rose 1989), which is characterized by an antibody response through a complex process involving activation of resting B cells, their proliferation, and differentiation into immunoglobulin-secreting cells (Rose 1989, Weetman 1991), takes place. Lymphokines as well as cell surface antigens and cell adhesion molecules play an important role in the regulation of this B cell response (Kishimoto et al. 1992, Or et al. 1994).

Antithyroid drug therapy induces marked changes in the proportion of activated T helper and T suppressor cells, which have been associated with the suppression of
autoantibody production (Tötterman et al. 1987). Methimazole also induces rapid changes in circulating T, B and NK cells (Karlsson & Tötterman 1988). In addition, many other studies have been reported in which the behaviour of T cells in GD is analyzed (DeGroot & Quintans 1989). In contrast, information on the immunocellular effects of antithyroid drugs on the effector phase of the immunological response mediated by B cells is still scanty, even though some B cell subsets such as CD5+ B and CD5 − B cells have been related to the production of low- and high-affinity autoantibodies in human autoimmune diseases (Casahi & Notkins 1989, Suzuki et al. 1990, Mantovani et al. 1993). To the best of our knowledge, only the distribution of CD5+ and CD5 − B cell subsets have been examined before and during treatment with thyrostatic agents (Iwatani et al. 1989, 1992). Nevertheless, in these studies it was not possible to ascertain whether the effects observed were caused by the action of antithyroid drugs on the immune system per se, or whether they were the consequence of a decline in thyroid function. The present paper describes a longitudinal study performed to analyze prospectively the in vivo effects of methimazole treatment on a large number of circulating B cell subsets, most of which have not been previously explored, in a group of 18 consecutive GD patients. For this purpose, the patients were followed-up for 6 months, a period of time in which 100% of patients become euthyroid (Okamura et al. 1987). As control groups, both healthy volunteers and GD patients in long-term remission were included in the study.

Patients and Methods

Patients

Thirty-eight GD patients enrolled consecutively in our outpatient clinic and 19 age- and sex-matched healthy volunteers were studied prospectively. Eighteen of the GD patients 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 four men, ranging in age from 20 to 59 years (mean ± s.d., 35 ± 11). These patients were studied before treatment (day 0), and 7, 14, 30, 90 and 180 days after starting methimazole treatment. The patients were initially treated with 30 mg (15 patients) or 40 mg (three patients) methimazole/day. After 1 month the methimazole dose was reduced by half, and maintained throughout the study (6 months). After another month, 50 µg levothyroxine was added to this regime in ten patients to maintain euthyroidism. The remaining 20 GD patients were in long-term clinical and biochemical remission; among them were 16 women and four men, ranging in age from 19 to 63 years (mean ± s.d., 38 ± 13). Remission, with an average of 3-1 years, was obtained by previous treatment with methimazole or 131I therapy; at the time of the study these subjects were taking no medication.

Diagnosis of GD was based on the presence of symptoms and signs of thyroid hyperfunction, diffuse goiter, occasional ophthalmopathy, diffuse and homogeneous uptake in thyroid gammagrapy with 99mTc, increased serum levels of thyroxine, tri-iodothyronine and elevated levels of antibodies to the thyrotrophin (TSH) receptor (TSH-binding inhibitory immunoglobulin; TBII).

Thyroid hormones and autoantibodies

Thyroid hormones and TSH levels 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 levels, the ability of antibodies to block the binding of 125I-labelled TSH to porcine thyroid membranes was measured by a radioimmunoassay using a commercial kit (TRAK Assay; Henning, Berlin, Germany). For the determination of anti-thyroid peroxidase (TPO) antibodies in serum, a solid-phase RIA was used (Henning). Serum levels of anti-thyroglobulin (TG) antibodies were measured by a two-step immunoradiometric assay (Cis Bio Int., Gif-sur-Yvette, France). In all cases, thyroid autoantibodies were studied in blood samples obtained simultaneously with respect to both those used for TBII and thyroid hormone serum 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. All samples were analyzed by direct immunofluorescence using triple staining combinations of monoclonal antibodies (MoAb) directly conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE) and the PE-cyanin 5 (PE-Cy5) fluorochrome tandem. The reactivity of the MoAbs used is shown in Table 1 and they were combined using the following triple stainings (FITC/PE/PE-Cy5): FMC7/CD5/CD19, CD22/CD25/CD19, CD22/CD23/CD19. The CD5, CD22, CD23 and CD25 MoAbs were purchased from Becton/Dickinson (San Jose, CA, USA), the FMC7 from Serotec (Kidlington, Oxford, UK) and the CD19 from Caltag Laboratories (San Francisco, CA, USA). Staining of cells was performed by incubating 200 µl peripheral blood containing between 0.5 and 1 × 10⁶ nucleated cells with each of the MoAb combinations shown above (10 min at room temperature). Erythrocytes were then lysed by incubating the stained sample (10 min at room temperature) with 2 ml FACS lysing solution/Tube (Becton/Dickinson). The FACSort flow cytometer (Becton/Dickinson) and the Lysys II software program (Becton/Dickinson) were used for data.
Table 1. Monoclonal antibodies, CD and characteristics of the B cell-associated surface molecules analyzed

<table>
<thead>
<tr>
<th>MoAb</th>
<th>CD</th>
<th>Cell populations defined and their function (molecular mass, kDa)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leu12</td>
<td>19</td>
<td>Pan B cells; member of the Ig superfamily associates with slgM; may function to regulate B cell adhesion (90)</td>
<td>Clark &amp; Lane (1991)</td>
</tr>
<tr>
<td>Leu 1</td>
<td>5</td>
<td>Activated B cells; production of low- and high-affinity antibodies (67)</td>
<td>Casali &amp; Notkins (1989)</td>
</tr>
<tr>
<td>FMC7</td>
<td>—</td>
<td>Mature B cell subset; present in an important proportion of peripheral blood B cells (105)</td>
<td>Ferro &amp; Zola (1990)</td>
</tr>
<tr>
<td>Leu20</td>
<td>23</td>
<td>Mature B cells; low-affinity receptor for IgE; may stimulate activated B cells; B cell growth and differentiation (45–50)</td>
<td>Clark &amp; Lane (1991)</td>
</tr>
<tr>
<td>Anti IL-2</td>
<td>25</td>
<td>Activated B cells; low-affinity receptor for IL-2 (55)</td>
<td>Yokota et al. (1988)</td>
</tr>
<tr>
<td>Leu14</td>
<td>22</td>
<td>Pan B cells; Ig superfamily member; may lower the threshold required for triggering B cells through their antigen receptor; B lymphocyte-restricted adhesion molecule (130–140)</td>
<td>Clark &amp; Lanier (1989)</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>Torres et al. (1992)</td>
</tr>
</tbody>
</table>

Pan = total; slgM = surface IgM.

Table 2. Serum thyroid hormone levels and thyroid antibodies in GD patients before (day 0), after treatment with methimazole (MMI) and in long-term remission. Values are means ± s.d.

<table>
<thead>
<tr>
<th>Patients</th>
<th>TT4 (nmol/l)</th>
<th>FT4 (pmol/l)</th>
<th>TT3 (nmol/l)</th>
<th>FT3 (pmol/l)</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)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n=18)</td>
<td>289 ± 44</td>
<td>69 ± 29</td>
<td>6.6 ± 1.7</td>
<td>20.7 ± 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 (day 0)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
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<tr>
<td>(n=18)</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 7</td>
<td>211 ± 55*</td>
<td>49 ± 13*</td>
<td>3.7 ± 1*</td>
<td>9.8 ± 3.6*</td>
<td>0.09 ± 0.05</td>
<td>57 ± 72</td>
<td>2600 ± 3229</td>
<td>164 ± 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>2775 ± 3356</td>
<td>207 ± 417</td>
</tr>
<tr>
<td>Day 30</td>
<td>147 ± 79*</td>
<td>26 ± 17*</td>
<td>2.7 ± 1.2*</td>
<td>7.6 ± 3.5*</td>
<td>0.27 ± 0.07</td>
<td>45 ± 32</td>
<td>1992 ± 2412</td>
<td>249 ± 464</td>
</tr>
<tr>
<td>Day 90</td>
<td>123 ± 91*</td>
<td>22 ± 23*</td>
<td>2.5 ± 1.7*</td>
<td>6.6 ± 6.1*</td>
<td>10 ± 31</td>
<td>39 ± 39</td>
<td>1369 ± 1573</td>
<td>283 ± 656</td>
</tr>
<tr>
<td>Day 180</td>
<td>92 ± 40*</td>
<td>14 ± 7*</td>
<td>1.6 ± 0.4*</td>
<td>4.3 ± 1*</td>
<td>6.9 ± 8.2†</td>
<td>18 ± 15</td>
<td>1896 ± 2826</td>
<td>31 ± 37</td>
</tr>
<tr>
<td>Long-term remission (n=20)</td>
<td>94 ± 33*</td>
<td>16 ± 6*</td>
<td>1.7 ± 0.3*</td>
<td>4.7 ± 1.3*</td>
<td>8.7 ± 14.2†</td>
<td>13 ± 23</td>
<td>3471 ± 3557</td>
<td>677 ± 1222</td>
</tr>
</tbody>
</table>

TT4 = Total thyroxine (normal range 51–141 nmol/l); FT4 = free thyroxine (normal range 10.4–23.4 pmol/l); TT3 = total tri-iodothyronine (normal range 1.2–2.8 nmol/l); FT3 = free tri-iodothyronine (normal range 2.3–6.9 pmol/l); TSH = thyrotrophin (normal range 0.15–5 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<0.05, *P<0.01 both with respect to hyperthyroid untreated GD patients (day 0).

acquisition and information on at least 15 000 events/tube was stored. For data analysis, the Paint-A-Gate plus software (Becton/Dickinson) was used according to previously described methods (Corrales et al. 1994). The absolute count of each lymphocyte subset was calculated by multiplying their percentage from the total peripheral blood lymphocytes by the peripheral blood lymphocyte count obtained using an automated cell counter (Coulter Corporation, Miami, FL, USA).

Statistical methods

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 ANOVA and in cases where ANOVA was statistically significant the cause of significance was explored by the Fisher least significant difference test.

Results

Table 2 shows the mean (± s.d.) serum levels of thyroid hormones, TSH, anti-TSH receptor antibodies and thyroid antibodies in GD patients before antithyroid drug therapy (day 0), during the course of treatment and in the remission phase. During methimazole therapy, the thyroid hormone serum levels declined progressively (P<0.001), reaching values similar (P≥0.05) to those found in GD patients in long-term remission after 90 days of therapy. In a similar way, from day 30 of treatment the TBII serum levels were not significantly different from those found in GD patients in long-term remission. Anti-thyroglobulin antibody serum levels reached normal values at day 180 of treatment, although some patients in long-term remission still showed increased values. By contrast, anti-TPO antibody serum levels, although reduced after methimazole therapy, never reached normal values, and were increased even in GD patients in long-term remission.
Table 3 Absolute number (× 10⁶/l) of the peripheral blood B lymphocyte subsets analyzed in untreated GD patients, in GD patients under treatment with methimazole (MMI), in GD patients in long-term remission and in controls. Data are expressed as means ± S.D.

<table>
<thead>
<tr>
<th>Patients</th>
<th>Total B (CD19+)</th>
<th>CD5⁺</th>
<th>CD5⁻</th>
<th>FMC7⁺</th>
<th>CD5⁺/FMC7⁺</th>
<th>CD23⁺</th>
<th>CD25⁺</th>
<th>CD22⁺</th>
<th>CD22⁺/CD25⁺</th>
</tr>
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<tbody>
<tr>
<td>Untreated (day 0)</td>
<td></td>
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</tr>
<tr>
<td>(n=18)</td>
<td>400 ± 168*†</td>
<td>223 ± 120*†</td>
<td>177 ± 107</td>
<td>342 ± 168*†</td>
<td>179 ± 103*†</td>
<td>299 ± 151*†</td>
<td>57 ± 36</td>
<td>395 ± 162*†</td>
<td>58 ± 37</td>
</tr>
<tr>
<td>Treated MMI</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>(n=18)</td>
<td>369 ± 180*†</td>
<td>207 ± 139*†</td>
<td>162 ± 88</td>
<td>284 ± 129*†</td>
<td>168 ± 97*†</td>
<td>278 ± 143*†</td>
<td>59 ± 48</td>
<td>376 ± 186*†</td>
<td>61 ± 47</td>
</tr>
<tr>
<td>Day 7</td>
<td>306 ± 152</td>
<td>185 ± 120*†</td>
<td>121 ± 59</td>
<td>289 ± 167*†</td>
<td>190 ± 138*†</td>
<td>234 ± 129*†</td>
<td>43 ± 35</td>
<td>297 ± 158</td>
<td>38 ± 36</td>
</tr>
<tr>
<td>Day 14</td>
<td>363 ± 138*†</td>
<td>197 ± 105*†</td>
<td>166 ± 99</td>
<td>351 ± 137*†</td>
<td>203 ± 121*†</td>
<td>258 ± 110*†</td>
<td>52 ± 48</td>
<td>355 ± 131*†</td>
<td>51 ± 47</td>
</tr>
<tr>
<td>Day 30</td>
<td>420 ± 194*†</td>
<td>188 ± 93*†</td>
<td>232 ± 155</td>
<td>376 ± 193*†</td>
<td>175 ± 95*†</td>
<td>323 ± 142*†</td>
<td>59 ± 50</td>
<td>432 ± 183*†</td>
<td>59 ± 49</td>
</tr>
<tr>
<td>Day 90</td>
<td>422 ± 97</td>
<td>147 ± 75*</td>
<td>195 ± 75</td>
<td>308 ± 77*</td>
<td>132 ± 63*</td>
<td>263 ± 98*</td>
<td>52 ± 29</td>
<td>363 ± 90*</td>
<td>53 ± 30</td>
</tr>
<tr>
<td>Day 180</td>
<td>324 ± 129</td>
<td>97 ± 72</td>
<td>138 ± 79</td>
<td>144 ± 112</td>
<td>59 ± 43</td>
<td>149 ± 92</td>
<td>53 ± 28</td>
<td>201 ± 139</td>
<td>46 ± 24</td>
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<tr>
<td>Long term remission</td>
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<tr>
<td>(n=20)</td>
<td>233 ± 112</td>
<td>55 ± 31</td>
<td>178 ± 100</td>
<td>194 ± 86</td>
<td>44 ± 31</td>
<td>134 ± 59</td>
<td>55 ± 32</td>
<td>225 ± 11</td>
<td>53 ± 31</td>
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<td>Controls</td>
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<tr>
<td>(n=19)</td>
<td>233 ± 112</td>
<td>55 ± 31</td>
<td>178 ± 100</td>
<td>194 ± 86</td>
<td>44 ± 31</td>
<td>134 ± 59</td>
<td>55 ± 32</td>
<td>225 ± 11</td>
<td>53 ± 31</td>
</tr>
</tbody>
</table>

*P<0.001 vs controls; †P<0.001 vs GD in long-term remission.
The absolute number of peripheral blood B cell subsets analyzed in the present study in both GD patients and healthy controls are shown in Table 3. The total number of B cells, as identified by the CD19 and CD22 antigens, was significantly increased in hyperthyroid untreated patients and at least during the first 90 days of methimazole treatment as compared with both healthy controls and GD patients in long-term remission \((P<0.001)\). This overall increase in the number of peripheral blood B cells was due to a significant increase \((P<0.001)\) of the CD5+, FMC7+, CD5+/FMC7+ and CD23+ B cell subsets. No significant differences were observed either prior to or during methimazole treatment as compared with healthy individuals and GD patients in long-term remission for the CD5− and CD25+ B cell subpopulations. The absolute numbers of all the B cell subsets analyzed before treatment (day 0) were not statistically different with respect to those observed during the whole follow-up period (days 7–180).

Figure 1 shows the percentage of the peripheral blood CD5+, FMC7+, CD5+/FMC7+ and CD23+ B cell subsets in healthy controls and GD patients. The percentages of CD5+, CD5+/FMC7+ and CD23+ B cell subsets were significantly higher \((P<0.001)\) in untreated GD patients with active disease and during the follow-up period than in the healthy subjects. In a similar way, the proportion of these three B cell subsets was significantly higher \((P<0.001)\) in GD patients before treatment and during the first month of therapy than in GD patients in long-term remission. From day 30 of methimazole treatment, only the CD23+ B cell subset remained significantly increased \((P<0.001, \text{at day 90})\) as compared with long-term remission patients. On comparing the proportion of these B cell subsets during the follow-up period it was observed that the CD5+ B cells were significantly higher \((P<0.001)\) on days 0, 7, 14 and 30 than after 6 months of therapy. In a similar way, the proportion of CD5+/FMC7+ B cells was significantly higher on day 14 of methimazole treatment as compared with the values found on day 180 \((P<0.001)\). By contrast, the values of both FMC7+ and CD23+ B cells were similar at all times during the follow-up period (day 0–180).

As compared with both healthy controls and GD patients in long-term remission, in GD patients before treatment and during the follow-up period the proportion of CD25+ B cells was significantly \((P<0.007)\) reduced (Fig. 2). Similarly, the percentage of peripheral blood CD5− B cells was significantly \((P<0.001)\) decreased in GD patients before treatment and during the first month of follow-up as compared with both healthy controls and GD patients in long-term remission. In addition, CD5− B
cells at 90 and 180 days of the follow-up period were significantly lower than in healthy controls \( P<0.001 \), although no statistically significant difference was detected with respect to the GD patients in long-term remission. When the patients followed longitudinally were compared, it was noted that the percentages of CD5 - B cells before therapy and during the first 30 days of methimazole were significantly lower \( P<0.001 \) than those found at the end of the period \( (\text{day } 180) \). The proportion of the remaining B cells presented in Fig. 2, analyzed during the different times after starting treatment with methimazole, was similar to those observed before treatment \( (\text{day } 0) \).

In order to explore whether the changes observed in the distribution of B cell subsets during the period of methimazole therapy might be induced directly by the effects of the antithyroid drug on the immune system \textit{per se} or whether they could be influenced by the progressive decline in the serum levels of thyroid hormones, the distribution of these lymphoid subsets in GD patients being treated with methimazole but still hyperthyroid \( (\text{day } 7) \) was compared with that of GD patients also treated with methimazole but now euthyroid \( (\text{day } 180) \). As shown in Fig. 3, the only differences observed were a lower \( P<0.001 \) proportion of CD5+ B cells and a higher \( P<0.001 \) percentage of CD5 - B cells in the euthyroid group as compared with the thyrotoxic patients. The possible influence of hyperthyroidism on the distribution of all the B cell subsets was also analyzed using a different methodological approach. Individual patients treated with methimazole for 14, 30 or 90 days were separated into two groups: group A \( (n=24) \) included samples from patients who were still hyperthyroid \( (\text{free thyroxine levels } 23.4 \text{ pmol/l and total thyroxine levels } 141 \text{ nmol/l}) \) and group B \( (n=20) \) included euthyroid patients. In a comparison between both groups (Fig. 4), significant differences were again found for the percentages of CD5+ and CD5 - B cells as well as for the proportion of CD5+/FMC7+ B cells. By contrast, no differences were observed for the remaining B cell subsets analyzed (results not shown).
Discussion

In the present study we found that GD patients before and during treatment with methimazole exhibited marked changes in the distribution of most of the peripheral blood B cell subsets analyzed with respect to both controls and GD patients in long-term remission. However, treatment with methimazole did not seem to have any important effects either on the absolute values or on the relative proportions of most of the B cell subsets studied, as indicated by a rather stable phenotypic expression of B cell surface antigens over the whole period of treatment analyzed as well as by the lack of differences between the active/untreated GD patients and those on treatment. Thus, during the follow-up period we only observed a gradual decrease in the proportion of both CD5+ and CD5+/FMC7+ B cells as well as an increase in the percentage of CD5−B cells found at the end of the treatment period.

Peripheral blood B cells expressing the CD5 antigen constitute 10–25% of circulating B lymphocytes in normal adults (Casali & Notkins 1989), a proportion much lower than that found in both our hyperthyroid untreated GD patients (55.8 ± 14.8%) and in the patients in long-term remission (41.2 ± 13.2%). These results confirm the striking increases in CD5+ B cells previously reported in

Figure 3. Comparison of the absolute and relative numbers of circulating B cell subsets between thyrotoxic (day 7) and euthyroid (day 180) GD patients during treatment with methimazole. Open circles show B cells (×10^6/l) and closed circles B cells (%). N.S. Not significant.
GD patients (Iwatani et al. 1989, Corrales et al. 1996). Nevertheless, the functional role of this increase in GD patients remains unknown. The CD5 protein functions as a receptor that specifically interacts with the CD72 cell-surface protein exclusive to B cells. For this reason, the CD5 antigen has been associated with cell to cell interactions where B lymphocytes are involved (van de Velde et al. 1991). Since antibodies specific for the CD72 antigen block the differentiation of B cells into antibody-secreting cells in the mouse (Yakura et al. 1981), the possibility exists that this molecule may play an important regulatory role in the B cell-associated immune response. Thus, CD5 expression in B cells would directly modulate the function of CD72. In vitro studies have shown that human CD5+ B cells produce polyclonal antibodies mainly of the IgM class (Casali & Notkins 1989), whereas the pathogenic antibodies in GD are IgG (Utiger 1995). It is therefore not surprising that in a previous work we were unable to find any relationship between the expansion of CD5+ B cells and the serum levels of TBI1 in hyperthyroid GD patients (Corrales et al. 1996). CD5+ B cells in GD could be involved in the production of low-affinity autoantibodies such as TPO and TG antibodies. In fact, in vitro studies have shown that CD5+ B cells make IgM antibodies against thyroglobulin (Casali et al. 1987) and, in vivo, Iwatani et al. (1989) have found a significant relationship between the percentage of peripheral blood CD5+ B cells and the serum levels of both TPO and TG autoantibodies. In our follow-up studies the levels of TPO and TG autoantibodies remained elevated in GD patients, as did the proportion of CD5+ B cells. Recently, Kasaian et al. (1992) have reported that a subset of CD5− B cells (CD5− /CD45RA+) produce IgG antibodies, opening the possibility that this could be the B cell subset involved in the production of the pathogenic TBI1 in GD patients. The low proportion of this B cell subset within the CD5− B cells (around 6%, Kasaian et al. 1992) would limit the value of the approach used in the present paper for the identification of this B cell subset. Nevertheless, we cannot exclude such a pathogenic role, even for CD5+ B cells, that could act as precursors and/or as mediators of other B cells producing antibodies with selective affinity.

In the present paper both absolute and relative numbers of the peripheral blood lymphocyte subsets are included, since they carry a different type of information. This is specially true if we consider that the expression of the CD5 molecule on B cells acts as a B cell–B cell bridge. Accordingly, the functional role of CD5+ B cells providing either a stimulatory or an inhibitory effect on other B lymphocytes, including those producing the pathogenic antibodies in GD patients, will depend among other factors on the proportion of these B cell subsets.

It has previously been reported that methimazole induces rapid changes in the proportion of peripheral blood-activated B cells (Karlsson & Törteman 1988) as well as a gradual decrease in the proportion of CD5+ B cells (Iwatani et al. 1989, Corrales et al. 1996) whereas the proportion of CD5− B cells remains relatively unchanged (Iwatani et al. 1989). Although these changes in CD5+ B cells have been shown to be correlated with the serum levels of thyroid hormones (Iwatani et al. 1989, Corrales et al. 1996), it is not clear whether they could be due to a direct effect of methimazole on immune cells or whether they are caused indirectly by the inhibition of thyroid hormone levels induced by the thyrostatic drug. Our results suggest that there is no direct relationship between methimazole treatment and the level of circulating CD5+ B cells; in contrast, these were found to have some relationship with thyroid hormone serum levels, suggesting that either directly or indirectly both variables are associated in GD patients during methimazole treatment. Further studies on healthy individuals made thyrotoxic by hormone ingestion would contribute to elucidating these questions.

Figure 4 Peripheral blood CD5+, CD5− and CD5+/FMC7+ B cells in hyperthyroid and euthyroid GD patients being treated with methimazole. Results are expressed as means (in the middle of the columns) ± S.D. *P<0.01 vs hyperthyroid patients.
Other changes observed in the present study regarding the distribution of B cell subsets observed in GD patients followed longitudinally during methimazole therapy were the increase in the absolute and relative numbers of CD23+ B cells and a decreased proportion of CD25+ B cells. This is the first time that increases in CD23+ B cells have been reported in GD. The functional properties of these cells are only partly known. CD23 (FC ε RIII) is the low-affinity receptor for IgE (Gordon 1991) that has a role in B cell growth and differentiation (Yokota et al. 1988), probably functioning as a receptor for stimuli that would act on activated B cells (Clark & Lane 1991). The CD25 antigen (Tac, interleukin-2 (IL-2) receptor subunit p55, IL-2R p55) is the low-affinity receptor for IL-2 (Clark & Lanier 1989). The low percentage of B cells expressing CD25 found in our GD patients is in agreement with previous reports (Eisenstein et al. 1988). The role of IL-2 in GD is still unclear, although it could influence the immune response by acting as a growth- and/or differentiation-stimulating factor for B cells (Muraguchi et al. 1985) and stimulating Ig secretion by B cells (Goldstein & Kim 1993). Therefore, the B cell subsets analyzed in the present study could potentially be involved in several steps of the immune response that gives rise to GD; however, their levels are not influenced by methimazole, at least during the whole period of our study.

Controversy still continues over the possible effect of antithyroid drugs on the immune process. While some authors report that in vitro methimazole has a direct effect on the immune system (McGregor et al. 1980, Wilson et al. 1988), other experiments employing more therapeutic concentrations of the drug argue against such a mode of action in both in vitro (Signore et al. 1985, Tötterman et al. 1987, Karlsson & Tötterman 1988) and in vivo studies (Wenzel & Lente 1984, Reinwein et al. 1993).

Our results show that circulating B cells in GD patients are influenced by the autoimmune disease itself rather than directly by methimazole. However, these experiments were performed in peripheral blood and caution is required in interpreting the results obtained on circulating B cells in order to elucidate the pathophysiological mechanisms of thyroid autoimmune disease (Weetman et al. 1985). Nevertheless, the immunocellular changes observed during an antigen-induced response could be detected in peripheral blood since B cells are assumed to emerge from the bone marrow, after which they enter into the circulation (Casali & Notkins 1989), upon activation, B cells recirculate, Ig-producing plasma cells usually being found in the bone marrow. Accordingly, GD although being an organ-specific disease (Weetman & McGregor 1994), shows an abnormal distribution of peripheral blood B cells. In addition, peripheral blood B cells produce thyroid autoantibodies, including anti-TSH receptor immunoglobulins of the IgG isotype, in a similar way to the B cells in the thyroid gland (Iwatan et al. 1987, Fan et al. 1994).

The absence of clear evidence for a direct effect of methimazole in vivo on peripheral blood B cells points to the existence of a different target as the site of action, such as the thyroid gland. Although methimazole does not appear to exert its immunomodulatory effect via any reduction in thyrotre hormone (HLA-DR) expression (Atwa et al. 1995), it may act by influencing intrathyroidal lymphocyte function (Weetman et al. 1984) or by inhibiting the release by thyrocytes of cytokines that mediate intrathyroidal B cell proliferation and/or differentiation, thus reducing the lymphocytic infiltrate in the gland (Weetman & McGregor 1994), and/or by controlling thyrotoxicosis (Volpé 1994).

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