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Methimazole treatment in Graves' disease: Behaviour of CD5+B lymphocytes and regulatory T cell subsets

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Abstract. – In the present study we analyzed some circulating lymphocyte subsets in eleven patients affected by Graves' disease before and after three and six months of methimazole treatment. Peripheral blood mononuclear cells were studied by a panel of monoclonal antibodies with single and double fluorescence cytometric analysis.

Our results demonstrated an increased percentage of CD5+B cells and HLADR+T lymphocytes at the beginning of the disease in comparison to the normal controls ($p < 0.001$), and a significant decrease after six months of treatment ($p < 0.01$ and $p < 0.05$, respectively). The CD4+ CD45RA+subset was significantly reduced in untreated Graves' patients in comparison to the normal group ($p < 0.01$), and increased towards normalization after six months of treatment. The significant modifications of lymphocyte subsets, as well as the reduction of thyroid autoantibodies, support a direct or mediated effect of methimazole on the immune system.

Key Words:

Lymphocyte subsets, Autoimmune thyroid diseases, Thyrostatic drugs.

Introduction

Autoimmune thyroid diseases (ATD) are generally considered to be caused by an immune imbalance. Many studies have been performed in order to specify the role of T and B cells, lymphokines and autoreactive clones in ATD. Modifications of thyroid infiltrating lymphocytes have been reported as the disease evolves and also with thyroid drug treatment[1]. Thyrostatic drugs (TDs) of the thioureylene type, such as propylthiouracil (PTU), methimazole (MM) and carbimazole (CM), are effective in humans. They inhibit the synthesis of thyroid hormones by blocking the incorporation of iodine into tyrosyl residues of thyroglobulin (TG) and the coupling of these iodotyrosyl residues to form iodothyronine, interfering with the oxidation of iodide ion and iodotyrosyl. PTU also inhibits the peripheral deiodination of thyroxine to triiodothyronine[2].

TDs inhibit the thyroid peroxidase (TPO) enzyme, influencing the TPO catalyzed iodination of thyroglobulin in vitro and in vivo[3].

In ATD (Graves' disease and thyroiditis) TDs induce remission of symptoms, decrease of T4, antithyroglobulin (aTG), and antithyroid microsomal fractions (aFM) antibody levels[4-6]. In addition the antithyroid treatment reduces the incidence of TSH binding inhibiting antibodies (TBIAb), present in about 90% of the untreated patients with Graves' disease[7].

These data supported both a direct and a mediated effect of TDs on the immune system: in fact, TDs could either interfere with the oxidative reactions of the antigen-presenting cell or modulate the thyrocyte activity by reducing the thyroid antigen expression in Graves' disease (GD)[8].

The inhibitory effect of MM on the occurrence of iodine-induced lymphocytic thyroiditis in the BB/Wor rat may be due to the lower antigenicity of the poorly iodinated TG following MM therapy[9]. The administration of thionamide to patients with GD may be associated with an increase in T suppressor cells and a decrease in activated T helper cells in the peripheral blood[10-11].

Similar modifications have been observed after addition of MM to lymphocyte cultures from untreated patients with GD[12].

Nevertheless, the view that TDs are immunosuppressive is controversial and the evidence deserves careful analysis.

In a previous study we analyzed CD5+B lymphocytes and regulatory T cells subsets in a group of patients affected by new-onset GD: we observed an important increase of CD5+B cells and a defect of the CD4+ CD45RA+ T lymphocytes[13]. In a further investigation we analyzed CD5+B cells in different forms of autoimmune thyroid diseases and we demonstrated a marked increase in both percentage and absolute number of CD5+B cells only in active GD[14].

Therefore, it was the purpose of the present study to analyze some circulating lymphocyte subsets, such as CD5+B cells, CD4+CD45RA+ T lymphocytes and activated T cells, as well as some biohumoral parameters, in a selected group of patients affected by GD before and during MM administration.

Materials and Methods

Patients and protocol

Eleven patients (7 females, 4 males), mean age 50 ± 8.1 years, affected by new-onset GD with ophthalmopathy were studied. The patients had typical signs and symptoms of hyperthyroidism: ophtalmopathy, palpable, soft and diffuse goitre, elevated serum T3 and T4 and very low or undetectable TSH levels, elevated incidence of anti-thyrotropin-receptor antibodies measured by a radio receptor assay (rTSH Ab RIA – Ares-Serono Diagnostici, Italy), and increased thyroid radioactive iodine uptake. No patient presented clinical evidence of infectious or other autoimmune diseases, nor was receiving any drug affecting immune function. After a baseline evaluation (T_0), all patients were treated with methimazole (30 mg/day). After 30 days, the dose has been reduced to 20 mg/daily for three months and to 15 mg/daily until the sixth month. During this period we did not associate other drugs (i.e. T3 or levothyroxin, or steroids) to avoid any interference of these drugs on lymphocyte function. However, we did not reveal any worsening of ophthalmopathy or hypothyroidism. The same parameters studied before treatment were monitored after three (T_1) and six months (T_2) of therapy.

Twenty healthy subjects matched for age and sex were evaluated as controls.

Hormonal and autoimmune parameters

Serum total T3, total T4 and TSH were measured by radioimmune assay (RIA), using commercially available kits (Baxter Immunoassay System). The normal range was: T3 = 1.32-2.9 nmol/l; T4 = 58-154 nmol/l; TSH = 0.5-4.5 mU/l.

Anti-thyroglobulin (TGAb) and anti-microsomal fraction autoantibodies (MFAb or TPOAb) were evaluated using passive haemagglutination (Thymume T, Thymume M respectively, Wellcome Kits), before and after three and six months of treatment.

Analysis of circulating lymphocyte subsets by cytometric evaluation

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hysopaque density gradient centrifugation. PBMC have been analyzed by a panel of monoclonal antibodies (MoAb) (Becton-Dickinson) tagged with either fluorescein isotiocyanate or phycoerithrin, i.e. aCD20, aCD3, aCD4, aCD8, aIL2R. Moreover, we evaluated the CD5+B cells, the HLA-DR+ T lymphocytes and the CD4+CD45RA+ subset with double fluorescence. These subpopulations have been expressed as percentages of total B cells, total T cells and CD4+ lymphocytes, respectively. The optimal dilution of monoclonal antibodies was established in preliminary experiments.

The cytometric analysis was performed on a FACS Analyzer (Becton-Dickinson). The cells were immediately applied to the flow without formaldehyde fixation. Cells were gated for lymphocyte characteristics to minimize the inclusion of other leukocytes or dead cells; 10,000 cells were counted in each analysis. Cells were scored as positive or negative staining, according to their fluorescence (red and green). The specific percentages of positive cells were obtained by subtracting the background fluorescence determined with fluorescein isothiocyanate or phycoerithrin-irrelevant mouse IgG, applied instead of the specific antibody.

Data analysis

Results were expressed as mean ± standard error (SE). To establish the statistical significance of observed differences non-parametric

Wilcoxon signed-rank test was used. P values less than 0.05 were considered significant.

Results

Hormones and autoantibodies levels are summarized in Figures 1 and 2.

Lymphocyte markers

White blood cells and total lymphocytes were in a normal range before and during treatment.

B cells were normal in the percentage and in absolute number, at the baseline evaluation. During the follow-up we did not observe any modification.

CD5+B cells, increased in untreated patients versus normal controls (45.3 ± 19.6% versus 19.5 ± 6.9) ($p< 0.001$), showed a reduction after three (36.8 ± 14.3%) and six months (27.6 ± 11.1%) of treatment (T_0 vs T_1: $p = 0.04$; T_0 vs T_2: $p = 0.01$). In particular, a decrease was present in 5 out of 11 patients after three months and in 8 out of 11 after six months of therapy (Figure 3).

Total T cells showed a normal percentage (T_0 = 70.1 7.7%; T_1 = 71.8 ± 6.5%; T_2 = 65.7 ± 7%) (normal range = 71.3 ± 6.1%).

The percentage of activated T lymphocytes (HLADR+ T cells) was significantly increased in comparison to the control group (16.4 ± 9.7% versus 4.7 ± 3.2%) ($p< 0.001$) before therapy. During the treatment the percentage decreased to 14.9 ± 7% after three months (p = n.s.) and to 9.2 ± 7% after six months of treatment (T_0 vs T_2, $p< 0.05$). A reduction was demonstrated in 4 out of 11 patients after three months and in 9 out of 11 after six months of therapy (Figure 4).

The percentage of CD4+ T cells was in the normal range and no significant differences were observed at T_1 and T_2.

The characterization of the CD4+CD45RA+ subset showed a significant decrease at baseline evaluation in comparison with the normal group (36.1 ± 14.7% versus 52.3 ± 6.5%) ($p< 0.01$). During the treatment this percentage increased to 39.3 ± 9.9% after three months and to 47.9 ± 14.7% after six months. The increase was demonstrated in 4 out of 11 patients after three months and in 8 out of 11 patients after six months (Figure 5).

The percentage of CD8+ T cells was in the normal range and no significant changes were observed during methimazole administration.

The CD4/CD8 ratio was normal both before (1.4 ± 0.5) and during treatment (1.5 ± 0.5 at T_1 and 1.4 ± 0.4 at T_2).

The expression of IL-2R on T cells was in the normal range (1.5 ± 1.6%): T_0 = 1 ± 0.8%; T_1 = 1.1 ±1.4%; T_2 = 1.2 ± 0.4%.

No statistical correlation between lymphocyte subsets, hormonal and autoantibody modifications was found at the different times of evaluation.

Discussion

Our results demonstrated significant modifications of lymphocyte subsets during MM treatment. CD5+B cells, increased at the beginning of the disease, as well as activated T lymphocytes (HLADR+T), significantly decreased; the CD4+CD45RA+ lymphocytes, reduced at the baseline evaluation, increased towards normalization.

A possible immunomodulating effect of MM is still being discussed. Some studies suggest a direct immunosuppressive effect of MM and PTU in the BB/Wor or August rat, susceptible to autoimmune thyroid disease[9]. The administration of MM to the BB/Wor lymphopenic rat with a very low number of cytotoxic/suppressor lymphocytes determines an increase of this subset and a reduced activation of T-helper cells, with improvement of lymphocyte thyroiditis[15]. Reinhardt et al[16] observed that low doses of MM induce a decrease of spontaneous lymphocytic thyroiditis in the BB/Wor rat, and that high doses of this drug reduce serum TGAb titers. MM may act on the TPO-iodine system by inhibiting the induction of free radicals and/or scavenging free radicals generated by the iodine/TPO/H_2O_2 system[16]. This activity could reduce the antigenicity of the thyroid peroxidase itself, which is identified as the microsomal antigen[17]. Thyroid peroxidase or microsomal antigen is the enzyme that catalyzes the iodination of TG. MM may attenuate the immune response by decreasing the iodination of TG so that the molecule becomes less immunogenic.

In humans, the drug seems to affect the composition of lymphocyte subsets. Some authors

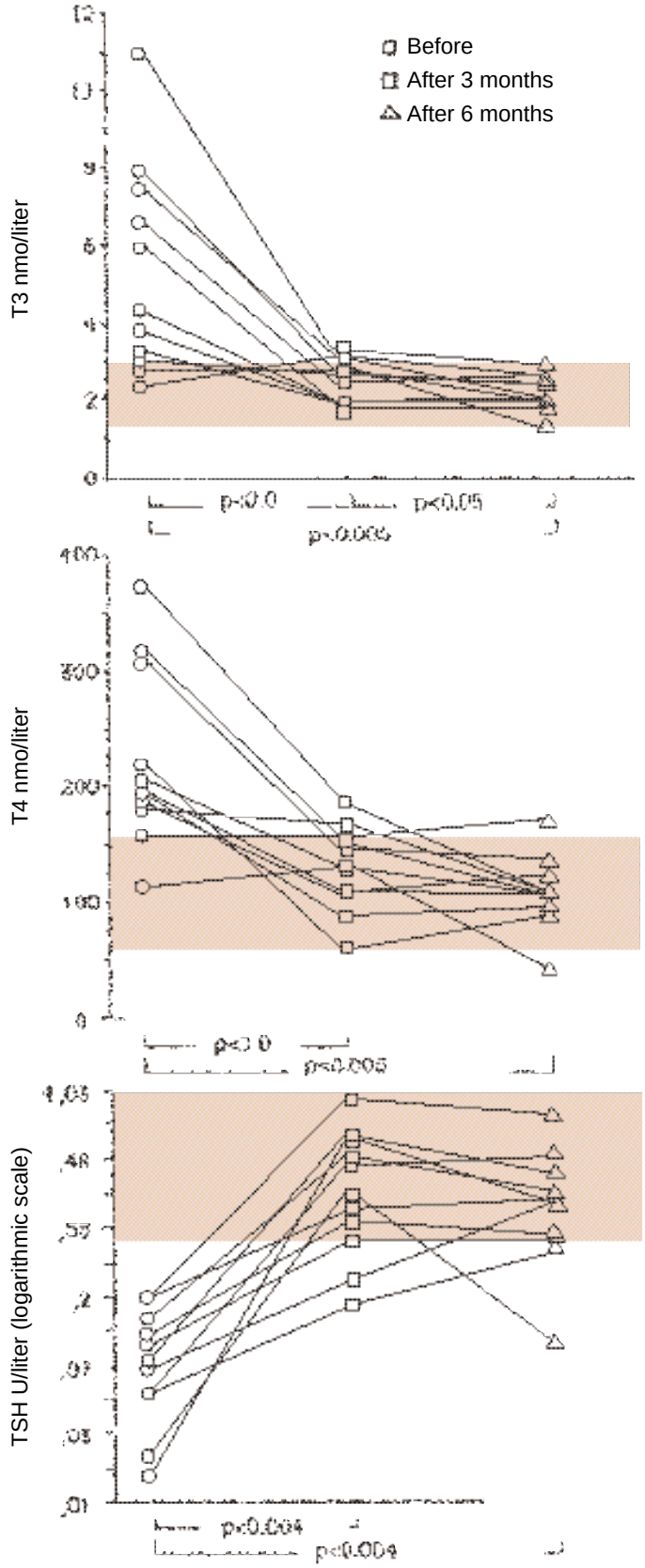

Figure 1. Behaviour of T3, T4, and TSH during MM treatment of Graves' disease.

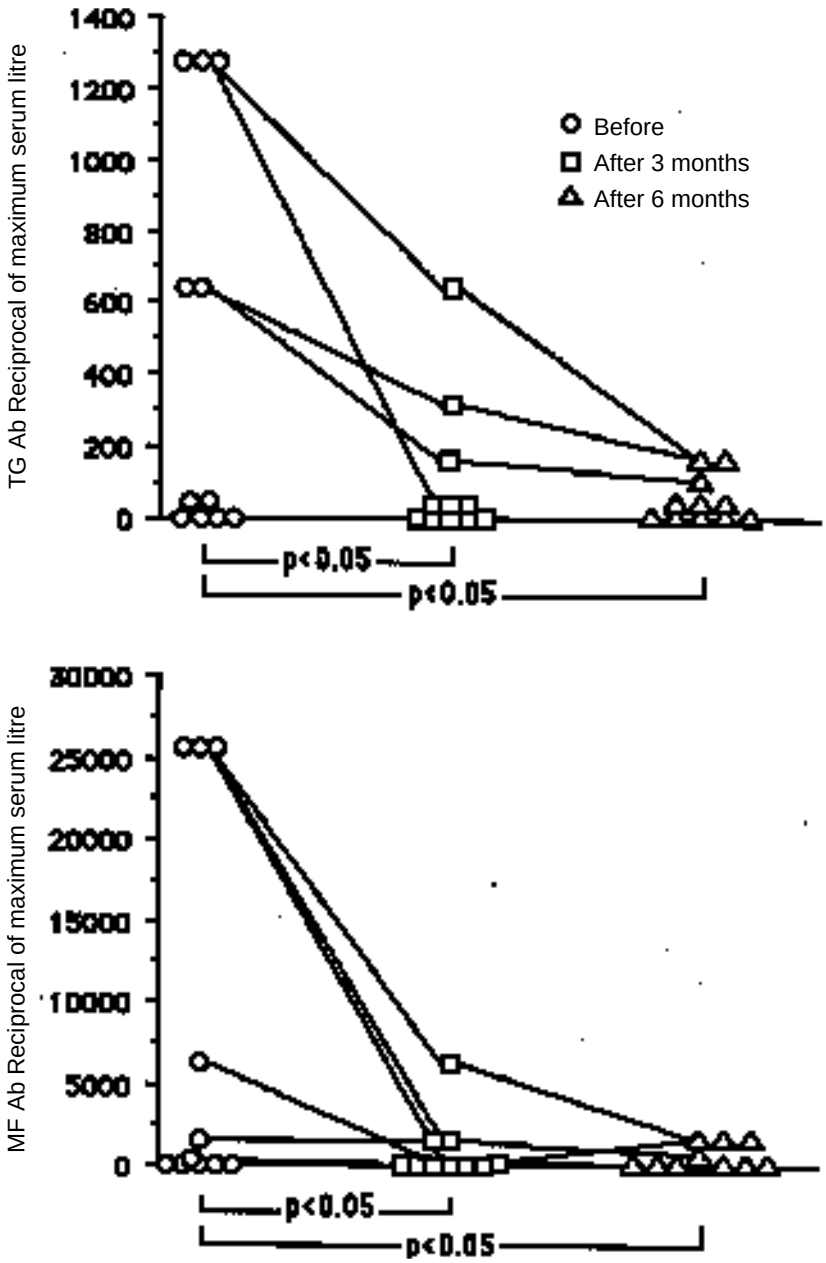

Figure 2. Behaviour of TG Ab and MF Ab during MM treatment of Graves' disease.

reported significant modifications in the activation of circulating lymphocyte functional subclasses upon methimazole[10]. Ohashi et al[1] demonstrated an increased percentage of HLADR+CD3+ cells, as well as HLADR+ CD4+ cells, wich seem to be independent on the treatment, in patients affected by GD; the percentage of HLADR+CD8+ cells was increased in euthyroid or hypothyroid subjects with Graves' disease following therapy, but was normal in hyperthyroid patients. Walfish et al, reported a reduction of HLADR+CD+ subset and an increase in the HLADR+CD8+ subset, in association with a reduction in serum T4 and T3 levels, in GD patients after PTU therapy[18]. Volpé et al postulated that TDs were acting directly on the thyroid cells reducing their hormone production and other activities[8] .

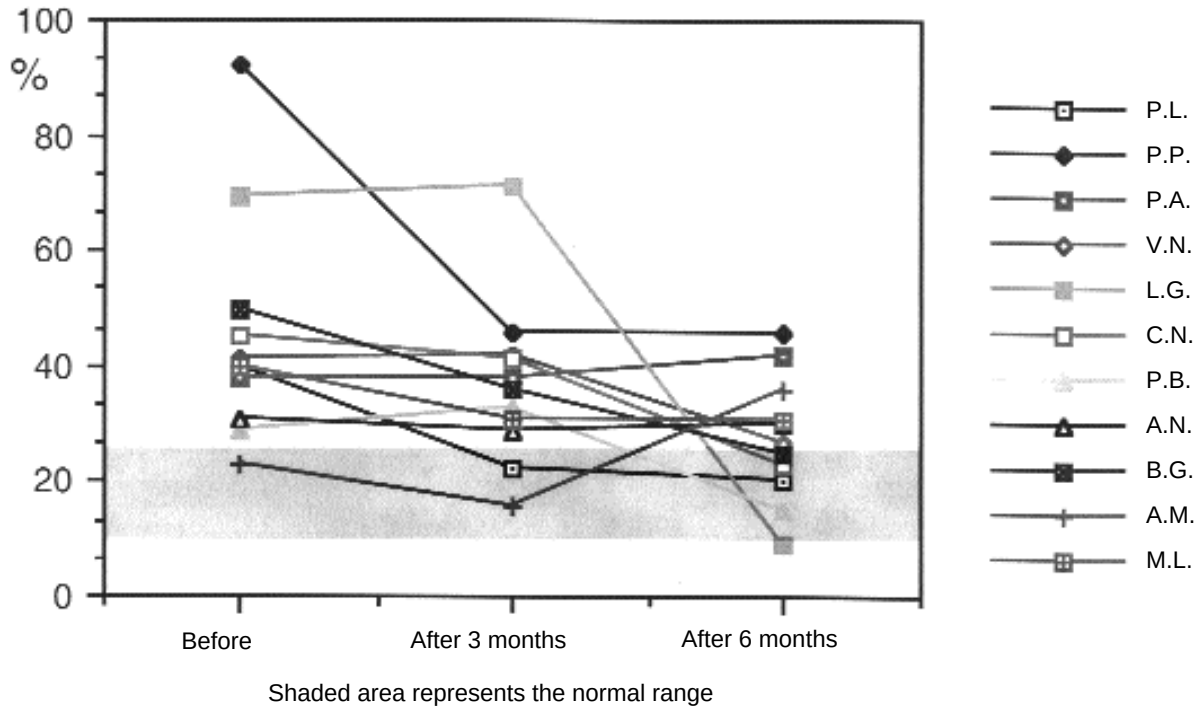

Figure 3. Behaviour of CD5+ B lymphocytes during MMI treatment of Graves' disease.

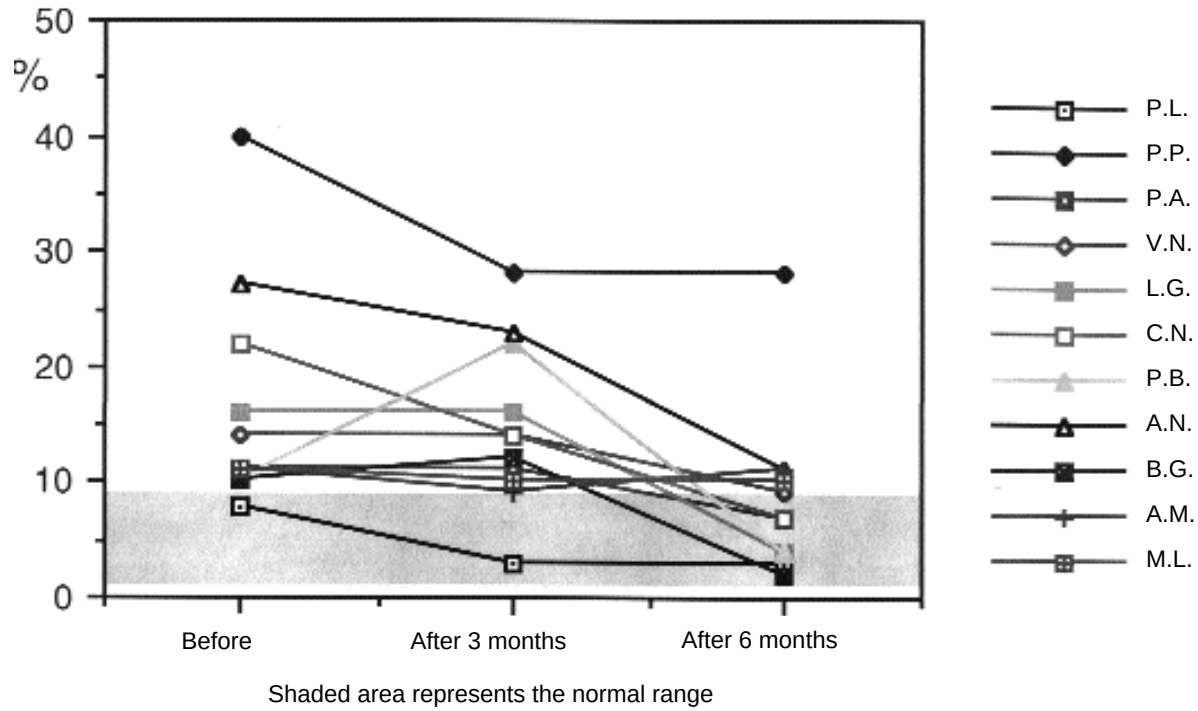

Figure 4. Behaviour of HLADR+ T lymphocytes during MMI treatment of Graves' disease.

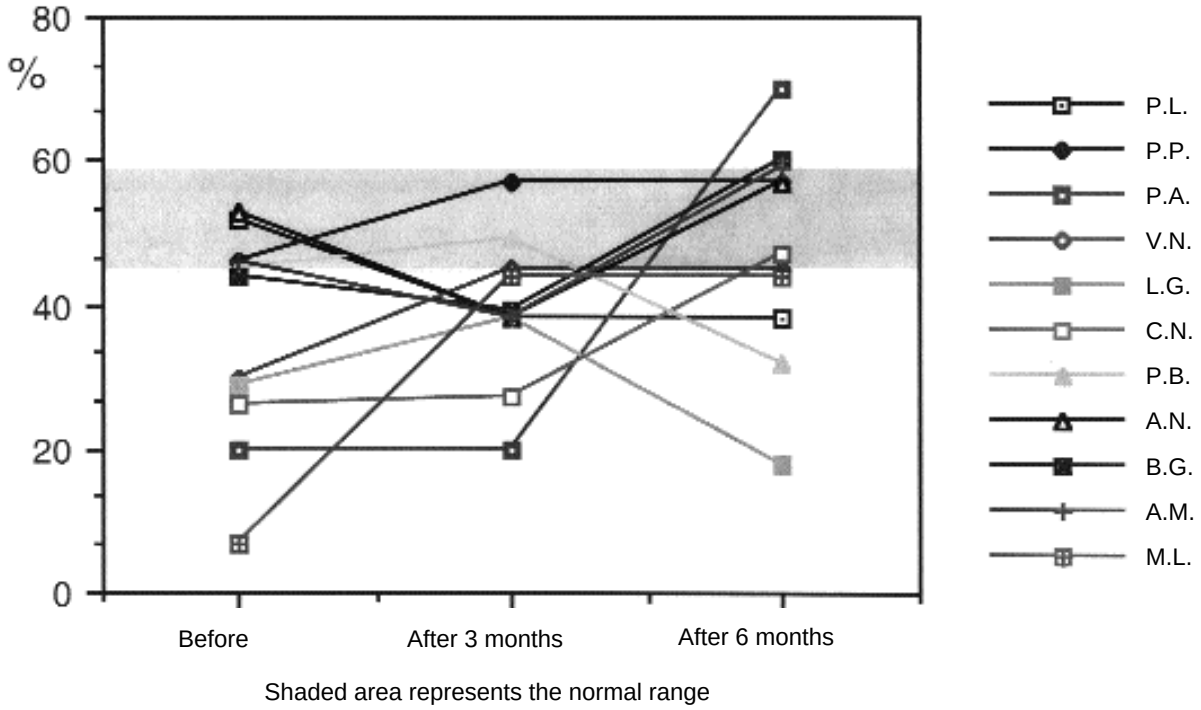

Figure 5. Behaviour of CD4+CD45RA+ lymphocytes during MMI treatment of Graves' disease.

Recent evidences demonstrated that there is an increased number and activation of CD8+CD11b+ cells, a reduction of soluble interleukin-2 receptor and that the thionamides interfere with thyrocyte expression of Class I antigen, interleukin-1, interleukin-6, prostaglandin E2 and heat-shock proteins: on the light of these results, Volpé confirms that the immunosuppressive effects of TDs are mediated through actions on the thyroid cell, modulating thyrocyte-immunocyte signaling[19-20].The present study demonstrated a CD5+B cell increase in GD at the baseline evaluation and a reduction of their value after three and six months of MM treatment.

It is well known that many reports have documented the increase of CD5+B cells in some autoimmune diseases[21-24].

Previous results of our group showed an increase of CD5+ B cells in new-onset untreated GD[13]. In a further investigation we analyzed CD5+ B cells in different forms of autoimmune thyroid diseases and we demonstrated a marked increase in both percentage and absolute number of CD5+ B cells only in active GD[14].

These cells, also defined B-1a cells, represent 10-25% of circulating and splenic lymphocytes and are the vast majority of B cells in human fetal spleen and cord blood. CD5+ B cells from healthy subjects are committed to the production of polyreactive antibodies[25,26]. Under the influence of environmental factors, these cells increase in number. At least some self-reactivities can be related to a significant enrichment in the CD5+ B cell population. The significant reduction of this subset after treatment supports a direct or mediated effect of methimazole on the immune system. However, we did not demonstrate a correlation between lymphocyte parameters and hormonal or antibody modifications, probably on the account of the low number of cases investigated.

Moreover we studied the immunoregulatory CD4+CD45RA+ cell subset. Indeed, functionally distinct T cell subsets can be separated using monoclonal antibodies to different isoforms of the leucocyte common antigen (CD45): in humans, antibodies to high (CD45RA) and low (CD45RO) molecular weight isoforms identify more or less recipro-

cal subpopulations. Our results showed that CD4+CD45RA+ lymphocytes were significantly reduced in untreated patients in comparison to the control group and increased towards normalization after six months of treatment. A defect of CD4+CD45RA+ cells has been described in some autoimmune diseases such as rheumatoid arthritis (RA)[27-29], systemic lupus erythematosus (SLE) and Sjogren's syndrome[30], Crohn's disease[31] and type 1 diabetes[32]. Kawakami et al demonstrated a reduced number of this subset in thyroid tissue of patients with GD[33] and similar results were obtained in peripheral blood lymphocytes from untreated Graves' disease[13]. All these data support the hypothesis that a decrease of such immunoregulatory subset, namely the suppressor-inducer CD4+ cells, determines a reduction of T suppressor cell function.

In our patients methimazole treatment resulted in a decrease of autoantibodies, CD5+ B cells and HLADR+ T lymphocytes; moreover, an increase of CD4+CD45RA+ subset was demonstrated. These results seem to confirm that methimazole nterferes on the immune response.

There are many ways whereby MM might effect either the immune system or the thyroid cells, but it is impossible to determine wheter the effects of this drug are specific for thyroid lymphocytes, aspecifically immunosuppressive, or secondary to changes in thyroid function. The reduction in thyroid hormone production and a lower thyroid antigenic expression MM-induced could lead to an improvement of the immune imbalance.

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