Introduction

Hashimoto's thyroiditis (HT) is considered as the most common organ-specific autoimmune disease. In women, the incidence of the disease is estimated to be around 3.5 cases/1000 people/year, while in men, this number is about 0.8 cases/1000 people/year (1). Morphologically, the disease is characterised by the progressive destruction of the gland, the replacement of its parenchyma by a lymphoid tissue, along with an ongoing alteration of thyroid functions that can ultimately lead to hypothyroidism. The diagnosis is usually made by the detection of thyroid auto antibodies against either thyroglobulin (anti Tg) or thyroperoxidase (anti TPO). Accumulated evidence over the recent years suggests that destructive thyroiditis results from T cell-mediated activation, especially originating from CD4+ T cells (2).

Two different functionally defined T cell clones have been identified so far, namely Th1 and Th2 cells. The Th1 subpopulation secretes proinflammatory cytokines, such as interleukin-2, interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α), and plays a major role in various destructive endocrine autoimmune diseases, such as in type I diabetes, but also in HT. Th1 cytokine-mediated biological actions are counter-balanced by Th2 cells that secrete anti-inflammatory cytokines, such as interleukin-4 (IL-4), IL-5, IL-6, IL-10, or IL-13 (3).

The main hypothesis of the present study is based on the idea that the proportion of the Th1/Th2 subsets of T cells in the peripheral blood could be altered in HT patients when compared to a group of control subjects. Consequently, testing functional subsets of this paper.
Colin et al

blood T cells could add additional clues about the knowledge and the outcome of the disease.

MATERIALS AND METHODS

HT patients and control healthy subjects

Twenty-three consecutive patients (n = 22 F / 1 M; mean age: 41 ± 3 years) with HT were seen in the outpatient clinic and asked to provide a blood sample to perform a cytometric evaluation of their peripheral lymphocytes (after informed consent). They were compared to 17 control healthy subjects (n = 11 F / 6 M; mean age: 37 ± 2 years) who were free of autoimmune disease, especially of thyroid autoimmunity. Among HT patients, eight were treated with levothyroxine (50 to 125 µg/day) at the time of investigation. They all had a heterogeneous tissue pattern compatible with chronic inflammation when analysed by thyroid ultrasound and plasma TPO ab levels higher than 600 IU/L. One patient had associated type 1 diabetes. Two others were known for having presented in the past at least one thyrotoxicosis crisis, likely in a context of hashitoxicosis, but were both euthyroid, substituted or not, at the time of the study.

Laboratory and thyroid function tests

All HT patients and normal healthy subjects were tested for eventual abnormalities in the haemogram. Plasma TSH and T4 levels were determined using commercially available kits (TSH: reference interval: 0.33 to 5.5 µUI/ml; Advia-Centaur, Bayer, Brussels; T4: reference interval: 11 to 24 pMol/ml; Advia-Centaur). TPO abs were measured using an ELISA kit (TPOAb ELISA, Biocode Liège Belgium). A concentration equal or higher than 600 IU/L was the criterion for entering patients in the study.

Flow cytometric analysis

The immunophenotyping of peripheral blood lymphocytes and the functional subsetting of CD3+ T and CD4+ T lymphocytes were carried out on a FACS Calibur™ analytical flow cytometer (BD-Biosciences Erembodegem Belgium). Daily procedures for instrument setting, compensation, and calibration were performed using Calibrite beads (BDB) and FacsComp software (BD-Biosciences). Daily performance was assessed by running fluorospheres calibration beads (Dako A/S Glostrup, Denmark).

Immunophenotyping

Immunophenotyping of lymphocytes was performed using a previously described method (4). It uses four color assays in one tube for flow cytometric immunophenotyping of lymphocytes. Briefly, blood specimens collected on K3EDTA were used within 2h following the sampling. Surface immunostaining of the sample was performed with fluorescein isothiocyanate (FITC)-conjugated CD8 (SK1, IgG1 isotype, BDB), FITC-conjugated CD19 (Sj25C1, IgG1 isotype, BDB), phycoerythrin (PE)-conjugated CD56 (MY 31, IgG1 isotype, BDB), peridinin chlorophyll A protein cyanin 5.5 (PerCP-Cy5.5)-conjugated CD3 (SK7, IgG1 isotype, BDB), allophycocyanine (APC)-conjugated CD4 (SK3, IgG1 isotype, BDB), in order to identify total T, T helper, T suppressor, NK cells, and B lymphocytes respectively. After staining for surface antigens, red cells were lysed and the samples centrifuged. After washing, white cells were suspended in PBS and run on the cytometer within two hours. Analysis of the flow cytometric data was performed using Attractor™ software (BD Biosciences), with an optimised Attractor set developed in our lab (5).

Functional lymphocyte subsetting

The method used was based on a technique described by Maino et al (6). Blood specimens collected on BD Vacutaine™ NH 143 I.U. (Sodium Heparin) were used within 2h following the sampling. The whole blood diluted in RPMI (Sigma St. Louis, MO, USA) was activated with phorbol myristate acetate (PMA) (Sigma) and ionomycin (Sigma) for 4 hours at 37 C in the presence of brefeldin A (GolgiPlug-BD-Pharmigen Erembodegem Belgium). Surface immunostaining of the samples was performed with PerCP-Cy5.5-conjugated CD3 and APC-conjugated CD4. After staining for the surface antigens, red cells were lysed and white cells were permeabilized using the BD FACS Permeabilizing Solution 2 (a buffered solution containing <15% formaldehyde, <50% diethylene glycol and a proprietary permeabilizing agent). The intracellular cytokines were then directly labelled using FITC-conjugated INF-γ (clone 25723.11, IgG1 isotype, BDB), and PE-conjugated IL-4 (clone 3010.211, IgG1 isotype, BDB) mabs. Analysis of the flow cytometric data was performed using CellQuest™ software (BD Biosciences), with thresholding on Fl3 positive cells. A logical gate combining CD3 positive cells and their scatter properties was used for studying the functional phenotype of CD3+ T-lymphocytes, or combining CD4+ T positive cells and the previously gated population for studying CD4+ T helper lymphocytes (Fig. 1).

Statistical analysis

Th1 and Th2 subsets are expressed as a percentage of either total CD 3+ T lymphocytes or CD4+ T lymphocytes. The significance of differences was reached for p< 0.05 using a nonparametric Wilcoxon signed-rank test.
Thyroid function tests in HT patients and in control healthy subjects

As shown in Table I, the thyroid function was considered as normal in control subjects. As expected, TPO abs were undetectable in this group, confirming that control subjects were free of thyroid autoimmune disease. In contrast, HT patients had high anti TPO ab plasma levels (>600 IU/L), and plasma TSH levels in the upper limit of the normal range, despite levothyroxine treatment (in 8 of 18 patients). T4 plasma levels were similar in both groups.

Flowcytometric analysis of peripheral lymphocytes in HT patients and in control healthy subjects

Data presented in Table II indicate that the lymphocyte subpopulations (T cells, B cells, NK cells, T CD4+ cells and T CD8+ cells) were the same in both groups, suggesting that there were no significant systemic immunological alterations in HT patients.

When the proportion of Th1 and Th2 subpopulations among CD3 lymphocytes and CD4 T cells was analysed between HT patients and control subjects, we did not observe significant differences for each independent parameter (Fig. 2). In contrast, when the Th1/Th2 ratio was calculated on CD3+ T lymphocytes, it came out as significantly increased in HT patients compared to the control group (p < 0.05). The same trend was observed on CD4 T cells, although the significance was not reached (Fig. 3).

**TABLE I - THYROID FUNCTION TESTS IN HT PATIENTS AND IN HEALTHY CONTROL SUBJECTS**

<table>
<thead>
<tr>
<th></th>
<th>TSH (mU/ml)</th>
<th>T4 (pg/ml)</th>
<th>TPO (AB Titer)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.32 ± 0.13</td>
<td>14.7 ± 0.58</td>
<td>0</td>
</tr>
<tr>
<td>Hashimoto</td>
<td>4.6 ± 1.4a</td>
<td>15.6 ± 0.7</td>
<td>&gt;600</td>
</tr>
</tbody>
</table>

*a TSH plasma levels were significantly higher in HT patients compared to control subjects (p < 0.05)*
DISCUSSION

We show in the present study that a routine immunophenotypic evaluation of lymphocyte subpopulations in HT patients does not discriminate them from healthy control subjects. As expected, no major alteration of the immune system can be detected in the peripheral blood of HT patients. These data are in accordance with those of Jansson et al who did not find differences in circulating lymphocyte subsets between thyrotoxic or hypothyroid female patients followed for postpartum thyroiditis compared to normal subjects (7). Two more recent papers also demonstrated that there is no significant difference in terms of T cell peripheral lymphocyte subpopulations between HT patients and control subjects (8, 9). This is likely due to the fact that peripheral T cells do not exactly reflect the profile of thyroid-infiltrating T cells (10). However, when an assay to identify specific Th1 and Th2 functional subsets is added to the flow cytometric evaluation of peripheral lymphocyte subpopulations, it comes out that the Th1/Th2 ratio is significantly different in HT patients compared to control subjects. This ratio is tipping in favour of the Th1 context and confirms many previous studies that demonstrated the predominance of Th1 T helper cells as pathogenic agents in destructive autoimmune thyroiditis (2, 11, 12). It is therefore likely that subtle changes can be found in peripheral lymphocytes of patients with thyroid autoimmune disease, as already reported in a previous study that showed a significant increase of activated T cells in patients with HT (9).

The detection of serum anti Tg or anti TPO abs remains undoubtedly the best and the less expensive way to diagnose HT. Indeed, virtually all patients have TPO abs at high levels. They at least indicate the autoimmune nature of the disease. However, they can be found in the general population without significance of clinically relevant thyroid disease (13). Furthermore, there is usually no direct relationship between TPO ab levels and the degree of thyroid destruction and thereby the likelihood to develop hypothyroidism. Although anti TPO abs can contribute to thyroid follicular destruction by complement fixation or by inhibition of thyroid functions, the mechanisms that lead to destructive thyroiditis are mainly T cell mediated (14). Hence, it is usually proposed that Th1 clones reactive to TPO or Tg antigens produce high levels of proinflammatory cytokines such as INF-γ or TNF-α that promote thyocyte apoptosis through FAS-FAS ligand interactions, or subcellular damages through NOSII activation (15, 16). In animal models of

<table>
<thead>
<tr>
<th>TABLE I - FLOWCYTOMETRIC ANALYSIS OF PERIPHERAL LYMPHOCYTES IN HT PATIENTS AND IN NORMAL HEALTHY SUBJECTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tot lymphocytes (nb/mm³)</td>
</tr>
<tr>
<td>-------------------------</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>Hashimoto</td>
</tr>
</tbody>
</table>

There was no significant difference for each analysed parameter between HT patients and control subjects.
autoimmune thyroiditis, such as the Biobreeding/Worcester rat model, the expression of Th1 cytokines is directly related to the degree of lymphocytic infiltration and therefore to the severity of thyroiditis (17). In our study, it was not possible to relate the Th1/Th2 ratio, either to the severity of the disease or to changes in the thyroid functional status, because we exclusively selected patients with high anti TPO ab levels, and because those who were hypothyroid were treated with levothyroxine. Nevertheless, the preponderance of the Th1 context, even evaluated in the peripheral blood, could correlate with the occurrence of hypothyroidism, as recently suggested (9).

The present study indicates that the Th1 context, as reflected by the Th1/Th2 ratio analysed in the peripheral blood, is indeed elevated in HT patients. It is therefore possible that this parameter could be used for further evaluation of HT patients, or of patients with other forms of destructive thyroiditis, such as post-partum thyroiditis. If one can show that this parameter could predict the outcome of the disease, this costly laboratory screening would become economically justified to better pick up patients prone to evolve toward hypothyroidism.

In conclusion, our data indicate that the Th1 context, even in peripheral lymphocytes, is prevailing in HT patients. Further investigations are in progress to evaluate whether flowcytometry could be used as a diagnostic tool to better understand the pathogeny and the outcome of destructive autoimmune thyroiditis.

Part of this work was presented at the 28th Annual Meeting of the European Thyroid Association, Göteborg, Sweden, September 2002.

Reprint requests to:
I. M. Colin, MD, PhD
Endocrinology Unit
Dpt. Internal Medicine
5, Av. B. Constantinople
B-7000 Mons, Belgium
colin@isto.ucl.ac.be

REFERENCES