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Thyroid-Infiltrating T Lymphocyte Subsets in Hashimoto's Thyroiditis*

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ABSTRACT. In Hashimoto's thyroiditis, the thyroid gland is infiltrated with immunocompetent lymphocytes. In this study we have used the fine needle aspiration technique to obtain thyroid-infiltrating lymphocytes from 11 HT patients for surface marker studies. The cells were characterized using conventional T and B cell markers as well as monoclonal antibodies (OKT) to different T cell subsets in a microscale immunofluorescence assay.

We observed a relative decrease in intrathyroidal suppressor phenotype T (OKT 8^+) cell numbers compared with peripheral blood (13% *vs.* 26%; $P < 0.01$ by Wilcoxon signed rank test). This resulted in an increased helper to suppressor T (OKT 4^+/OKT 8^+) cell ratio (4.7 *vs.* 2.1; $P < 0.01$). Within the gland, a significant local accumulation of B cells was also registered (27% *vs.* 12%; $P < 0.01$). As to circulating lymphocyte subsets, no differences were found between 18 Hashimoto's thyroiditis patients and 26 normal subjects.

Our results are compatible with a local synthesis of thyroid-directed antibodies and emphasize the importance of studying the local immunity in organ-specific autoimmune disease. (*J Clin Endocrinol Metab* **56:** 1164, 1983)

HASHIMOTO'S thyroiditis (HT) is regarded as an organ-specific autoimmune disease (1). Recent immunopathogenetic considerations have focused on possible changes in the immunoregulatory function of different T lymphocyte subsets in this disease. In analogy with several animal models (1), it has been hypothesized that a selective decrease in antigen-specific suppressor T cell function could permit the expansion of thyroid antigen-directed antibody-producing clones of B cells (2). Although some experimental evidence (3) has been presented for this mechanism in man, more studies are needed at the antigen-specific cellular level.

Most studies on the types and functions of lymphocytes in HT have been restricted to an analysis of peripheral blood cells. Using conventional lymphocyte markers, several workers demonstrated normal proportions of circulating T and B cells in these patients (4–7). However, in HT, the pathological process is localized to the thyroid gland, which is infiltrated with immunocompetent cells (8, 9). Using the fine needle aspiration biopsy technique combined with conventional T and B cell markers, we have previously demonstrated a relative enrichment of B lymphocytes in the thyroid glands of patients with juvenile autoimmune thyroiditis (10), HT (6), and Graves' disease (GD) (6). On the other hand, in patients with de Quervain's subacute thyroiditis, a disease believed to have a viral etiology, the vast majority of the thyroid-infiltrating cells were T lymphocytes (6).

The introduction of antihuman monoclonal mouse hybridoma antibodies has allowed the phenotypic identification of functional T cell subsets, such as helper/inducer cells and suppressor/cytotoxic cells (11). With the use of such antibodies it was recently demonstrated that patients with HT as well as GD have a decreased number of circulating suppressor T cells (12, 13). In the present study we have applied the fine needle aspiration technique to obtain thyroid-infiltrating lymphocytes for characterization with monoclonal antibodies to T cell subsets. In HT, the subset distribution of thyroid-infiltrating lymphocytes differed from that in peripheral blood. A highly significant decrease in the proportion of thyroid-infiltrating suppressor cells was demonstrated, while no difference was found in the subset distribution of circulating lymphocytes between HT patients and healthy controls.

Materials and Methods

Patients and control subjects

Eighteen females with HT (mean age, 45 yr; range, 19–62 yr) were studied. Routine fine needle biopsy of the thyroid

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gland was performed in all patients and showed the typical picture of lymphocytic thyroiditis in every case (14). The diagnosis of autoimmune thyroiditis was further verified by the demonstration of serum antimicrosomal (MCHA) and/or antithyroglobulin antibodies using passive hemagglutination techniques (Thymume-M and Thymume-T, Wellcome, Beckenham, England). All 18 patients were positive for MCHA, and 10 of the 18 had titer levels of 1:25,600 or more. Eleven patients had antithyroglobulin antibodies. Thyroid function was assessed by T_4, resin T_3 uptake, and TSH measurements in routine use at the Department of Clinical Chemistry at our hospital. The reference range for the calculated free T_4 index (FT_4I) was 67–153 nmol/liter, and that for TSH was 8 mU/liter or less. Ten patients received no thyroid hormone treatment. Of these, 8 were overtly hypothyroid (FT_4I, <67 nmol/liter; TSH, >8 mU/liter), while 2 patients had a low normal FT_4I and a slightly elevated TSH level. The remaining 8 patients had been treated with T_4 to euthyroidism for at least 1 yr. The peripheral white mononuclear cell counts were normal in all 18 HT cases. The control subjects consisted of 26 healthy persons (22 women and 4 men; mean age, 42 yr; range, 22–65 yr) negative for circulating thyroid antibodies. They had no signs, symptoms, or history of thyroid or other autoimmune diseases.

Collection and preparation of thyroid-infiltrating lymphocytes

Informed consent was obtained from all of the patients. Using a needle 0.7 mm in outer diameter, both thyroid lobes were punctured. In each patient, two or three repetitive aspirations were performed. The aspirates were diluted in 1 ml of a medium consisting of PBS (pH 7.4) with 0.5% bovine albumin, preservative-free heparin (12.5 IU/ml), and 2.5 mM Hepes buffer (Sigma, St. Louis, MO). A cytocentrifuge preparation from each aspirate and a peripheral blood smear was stained with May-Gruenwald-Giemsa. The degree of blood contamination was calculated as follows:

$$\text{index of blood contamination (IC)} = \frac{\text{lymphocytes/polymorphonuclear leukocytes in thyroid aspirates}}{\text{lymphocytes/polymorphonuclear leukocytes in peripheral blood}}$$

Aspirates with an IC less than 3.0 were not considered representative (10) and were not used for further analyses. Acceptable aspirates from the same subject were pooled, and the mononuclear white cells were separated from thyroid cells by density centrifugation over Ficoll-Isopaque (15) in agarose-coated 3-ml glass tubes. It was previously shown that no subclass-specific cell losses take place during this purification (10).

Preparation of blood lymphocytes

Ten milliliters of heparinized blood was fractionated by density centrifugation over Ficoll-Isopaque, and the lymphocytes were collected at the interface (15).

Identification of T cell subsets

Murine monoclonal antibodies (Ortho Pharmaceutical Co., Raritan, NJ, USA) specific for helper/inducer (OKT 4) (16) and suppressor/cytotoxic (OKT 8) (17) T lymphocyte subsets as well as total peripheral T cells (OKT 3) (18) were used in an indirect immunofluorescence assay (see below). In normal individuals, OKT 3 reacts with 95–100%, OKT 4 reacts with 55–65%, and OKT 8 reacts with 25–35% of peripheral T lymphocytes (11). T cells were also quantitated by a rosetting technique using 2-amino-ethyl-isothiouronium bromide hydrobromide-treated sheep erythrocytes (E) (19).

For the fine needle aspirate cells, a microscale technique was employed. A minimum of 30,000 cells was needed for the E-rosette assay and 90,000 cells were needed in the fluorescence staining. In preliminary studies no difference could be demonstrated between the microscale assay and a conventional assay using 200,000 cells in the E-rosette test and 1×10^6 cells in the immunofluorescence tests. Thus, the conventional technique was used for peripheral blood lymphocytes. The cell number was adjusted to 6×10^6/ml in PBS. The cells were incubated with the appropriate OKT antibodies (1:10 dilution of the commercial stock) in microtiter wells at 4 C for 30 min. The second layer was a fluorescein-conjugated sheep antimouse immunoglobulin antiserum (SBL, Solna, Sweden) diluted 1:40. No background staining was seen with this antiserum alone (data not shown). The washed cell suspension was mounted on microscopic slides in buffered glycerol. Two hundred lymphoid cells were counted, and the proportion of fluorescent cells was determined in a Leitz Orthoplan microscope (E. Leitz, Rockleigh, NJ) equipped with a ploemopaque epicondenser (immersion objective, 63 ×/1.30; ocular, ×4). The laboratory procedure was performed without knowledge of the source of the specimen.

Identification of B cells

Mononuclear cells carrying surface immunoglobulin (S-Ig) were stained by direct immunofluorescence using $F(ab')_2$ fragments of a fluorescein-conjugated polyvalent goat antihuman immunoglobulin (Kallestad, Chaska, MN) diluted 1:4.

Statistics

The Wilcoxon nonparametric tests for unpaired and paired data and Spearman's rank correlation were used for determination of significance when appropriate.

Results

The relative distribution of peripheral blood lymphocyte subclasses in HT patients and controls are presented in Table 1. No significant differences in the distribution of marker-carrying cells were observed between patients and controls, nor did patients presenting with hypothyroidism (FT_4I, <67 nmol/liter; TSH, ≥8 mU/liter) or high titers of MCHA (MCHA, ≥1/25,600) differ in this respect when compared with the controls or the rest of the patients (data not shown). A weak positive correlation was found between circulating $S\text{-}Ig^+$ cell levels and serum MCHA titers ($r_s = 0.511$; $P < 0.05$; by Spearman's rank correlation). There was no correlation between

TABLE 1. Circulating lymphocyte subpopulations (percentages) in HT patients and controls

Lymphocyte subset	HT patients (n = 18)	Normal controls (n = 26)
S-Ig$^+$	13 ± 6	16 ± 9
E$^+$	69 ± 11	68 ± 16
OKT 3$^+$	73 ± 10	73 ± 12
OKT 4$^+$	52 ± 7	53 ± 10
OKT 8$^+$	25 ± 4	28 ± 8
OKT 4$^+$: OKT 8$^+$ cell ratio	2.1 ± 0.3	2.0 ± 0.7

The results are given as the mean ± SD.

MCHA titers and other cell subset levels.

The ratio between phenotypically helper T cells (OKT 4$^+$) and suppressor/cytotoxic T cells (OKT 8$^+$) in peripheral blood was calculated in each subject to correct for errors that could be due to an altered percentage of total T cells. There was no difference in the OKT 4$^+$ to OKT 8$^+$ cell ratio between controls (mean ± SD, 2.0 ± 0.7) and HT patients (2.1 ± 0.3). The ratio of patients with hypothyroidism or high MCHA titers (≥1:25,600) did not differ from the controls or the rest of the patients. No correlation was seen between the age of the patients and this ratio (data not shown).

Representative thyroid fine needle aspirates were obtained in 11 patients. The lymphocyte yield ranged from 0.24–1.6 million cells (mean, 0.66 million), and the index of blood contamination ranged from 7–152 (mean, 35). The proportions of peripheral blood lymphocyte subsets in these patients did not differ from those in the whole group of 18 HT patients. Among the thyroid-infiltrating cells (Fig. 1), a relative decrease in OKT 8$^+$ cells (mean ± SD, 13 ± 6%) together with an increase in S-Ig$^+$ cells (27 ± 6%) were registered when compared to blood levels [26 ± 5% ($P < 0.01$) and 12 ± 4% ($P < 0.01$), respectively; by Wilcoxon signed rank test]. The percentages of intrathyroidal OKT 4$^+$ cells did not differ from those in peripheral blood (50 ± 6% *vs.* 53 ± 7%). The proportion of OKT 3$^+$ (total mature T) lymphocytes in thyroid aspirates was lower than that in blood (63 ± 5% *vs.* 75 ± 9%; $P < 0.02$). In accordance with this, a similar difference was observed in the percentages of E$^+$ cells (53 ± 7% *vs.* 73 ± 11%; $P < 0.01$). The OKT 4$^+$ to OKT 8$^+$ cell ratios were higher in the thyroid aspirates than in blood (4.7 ± 1.7 *vs.* 2.1 ± 0.4; $P < 0.01$). This was mainly due to the difference in OKT 8$^+$ cell levels. There was no correlation between serum MCHA titers and intrathyroidal S-Ig$^+$ cell levels ($r_s = 0.080$) or OKT 8$^+$ cell level ($r_s = 0.159$).

Discussion

The introduction of human lymphocyte-directed monoclonal mouse hybridoma antibodies (11) has made it possible to quantitate different subsets of T lymphocytes with simple immunofluorescence methods. Such antibodies recognize surface molecules specific for, *e.g.*, helper (OKT 4$^+$) and suppressor/cytotoxic (OKT 8$^+$) cells important in the regulation of B cell differentiation and killer T cell generation. It should, however, be remembered that the mere expression of these surface antigens is not necessarily connected to the functional state of the cell. Both OKT 4$^+$ and 8$^+$ cells are activated by alloantigens and T mitogens, whereas only 4$^+$ cells will proliferate in response to soluble antigen or autologous cells in mixed lymphocyte culture (20). In the effector phase, both 4$^+$ and 8$^+$ cells release lymphokines like interleukin-2, colony-stimulating factor, and γ-interferon (20). In the primary mixed lymphocyte culture, the vast majority of cytotoxic effector cells generated are 8$^+$ cells, with only a few lytic 4$^+$ cells (16). Interestingly, the activating and target alloantigens for OKT 8$^+$ cytotoxic cells are class I (HLA-A,B) antigens, whereas 4$^+$ killer cells recognize class II (Ia-like) antigens (20). Nearly all helper T cell activity has been found within the OKT 4$^+$ population (16).

In the present study, important differences were observed between circulating and thyroid-infiltrating lymphocyte subsets. In the thyroid infiltrates, relatively few OKT 8$^+$ cells were seen. This results in a higher OKT 4$^+$

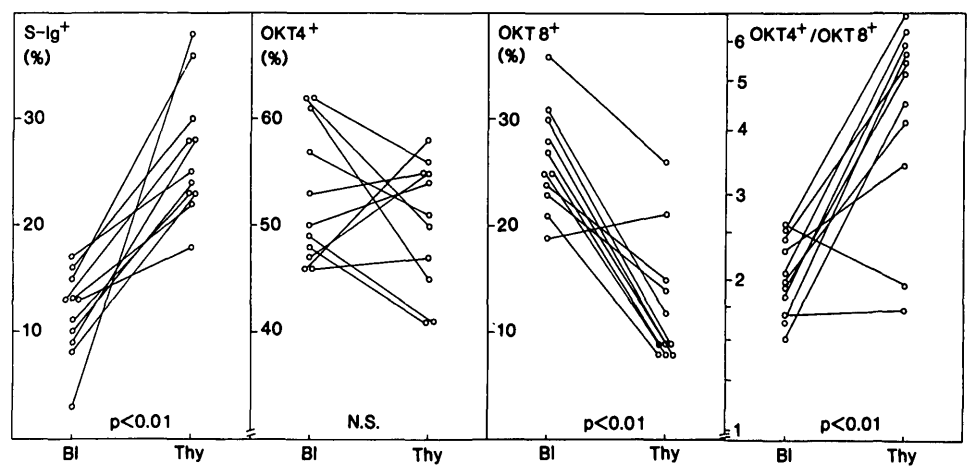

FIG. 1. Lymphocyte subsets (percentages) and OKT 4$^+$ to OKT 8$^+$ cell ratios in peripheral blood (Bl) and thyroid (Thy) aspirates from HT patients (n = 11). The Wilcoxon nonparametric signed rank test was used for statistical comparison.

to OKT 8^+ cell ratio in the thyroid gland than in peripheral blood. Taken together with the higher percentage of B cells within the thyroid, these data are in accordance with a high rate local synthesis of thyroid-directed antibodies. The present findings do not define the antigenic specificity or function of different thyroid-infiltrating lymphocyte subsets. However, we have previously demonstrated a highly significant (up to 61-fold) increase in the frequency of thyroglobulin-binding B and T cells in the gland compared to that in blood (6). Further, it was recently shown that thyroid-infiltrating lymphocytes from patients with HT have the capacity to produce Ig, including antithyroglobulin and microsomal antibodies *in vitro* (21, 22). The thyroid-infiltrating lymphocyte subset distribution observed here would also support an antibody-mediated or antibody-dependent mechanism in thyroid destruction. In accordance, we were unable to demonstrate an accumulation of thyroid antigen-reactive T cells in the thyroid glands of HT patients (10).

In the present study on HT patients, we found no differences in the proportion of circulating total peripheral (OKT 3^+), helper/inducer (OKT 4^+), or suppressor/cytotoxic (OKT 8^+) T lymphocytes compared to that in normal controls, nor did a subdivision of the patients, based on thyroid function or titer of antithyroid antibodies, indicate any alterations from normal. Our results are in contrast to those reported by Thielemans *et al.* (12). These investigators found a significant decrease in circulating OKT 8^+ cells in patients with asymptomatic thyroiditis, primary hypothyroidism, and GD, but there was no correlation to the titer of antithyroid antibodies or thyroid function. Sridama *et al.* (13) recently reported a slight mean reduction in OKT 8^+ cells in HT, but the results were widely distributed, and many of the patients had normal numbers of OKT 8^+ cells. The influence of variable degrees of disease activity was questioned by the investigators. This assumption is, however, not supported by our results or those of Thielemans *et al.* (12).

It was recently demonstrated that some patients with autoimmune thyroid disease may have autoantibodies specific for T lymphocytes and, preferentially, the OKT 8^+ T cell subset (23, 24). One explanation for the conflicting data on circulating suppressor T cells in HT could be that different numbers of such patients were included in the studies. However, the majority of patients seem to have a normal number of circulating suppressor T cells independent of their disease activity. If there is an abnormal suppressor T cell function in this organ-specific disease, it is expected to be selective and thyroid antigen specific.

In conclusion, we have found that thyroid aspirates from HT patients contain higher relative numbers of B lymphocytes and fewer suppressor/cytotoxic (OKT 8^+) T lymphocytes compared to the patients' peripheral blood. This is compatible with an effective local synthesis of thyroid-directed autoantibodies. We were not able to demonstrate any abnormalities in the circulating lymphocyte subset distribution in HT patients. Further studies on thyroid-infiltrating lymphocytes should increase our knowledge of the pathogenesis of HT and may also be of help in refining the classification of different types of thyroiditis.

Note Added in Proof

Recently Wall *et al.* (J Clin Endocrinol Metab 1983:56:164) also reported that circulating lymphocyte subsets were normal in HT. In contrast to our results, they found a normal subset distribution among intrathyroidal HT lymphocytes ($30–50 \times 10^3$) obtained by fine needle aspiration. However, the same group (Ann Endocrinol 1982:43:65A (abstract)) later confirmed our present findings when thyroid specimens obtained by surgery were analyzed. The authors themselves questioned the validity of their fine needle aspiration technique. In our hands, 10- to 20-fold higher cell numbers were needed and it was necessary to apply 5–6 monoclonal antibodies for indirect IFL suspension staining. Finally, in our view contamination with thyroid parenchymal cells (5–15%) after one-step density purification of the aspirates does not seem to represent a problem as was expected by Wall *et al.* The use of lymphocyte-specific monoclonal antibodies allows the correct quantification of relative lymphocyte subset frequencies.

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