Original Article

Cytologic, Flow Cytometry, and Molecular Assessment of Lymphoid Infiltrate in Fine-Needle Cytology Samples of Hashimoto Thyroiditis

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BACKGROUND: The thyroidal lymphoid infiltrate (TLI) in Hashimoto thyroiditis (HT) represents the substrate from which thyroid lymphoma may arise. The objective of the current study was to classify the TLI in HT by comparing the cytologic features with flow cytometry (FC) data and evaluating the κ/λ light chain ratio and its molecular assessment. **METHODS:** Fine-needle aspiration cytology (FNAC) was performed in 34 patients with HT with nodular or diffuse palpable enlargement of the gland. Two or 3 passes were performed to prepare traditional smears, FC, and immunophenotyping, and RNAlater suspensions for molecular assessment. FC was performed using the following antibodies: CD3, CD5, CD4, CD8, CD10, CD19, and κ and λ light chains. In 4 cases, high molecular weight DNA was extracted and used for polymerase chain reaction (PCR) to amplify the variable diversity joining region of the heavy chain immunoglobulin (Ig) genes (IgH). Statistical analysis was performed to evaluate possible associations between clinical ultrasound presentation, cytologic pattern, and TLI phenotype. Light chain expression was evaluated as the percentage of the expressing cells (≤20% and >20%) and as the κ/λ ratio. **RESULTS:** Smears were classified as "lymphocytic," "lymph node-like," or "mixed." FC demonstrated T cells (CD3 positive [+], CD5+) in all cases, and T cells and B cell (CD19+, CD10+/-) lymphocytes in 22 cases. Light chains were expressed in 30 cases (in <20% of the gated cells in 13 cases and in >20% of the gated cells in 17 cases). Five cases demonstrated small κ/λ ratio imbalances and PCR analysis demonstrated diffuse bands in the gel and Gaussian curves at the heteroduplex. Statistical analysis indicated significant associations between the "lymphocytic" pattern and T-cell phenotype and between the "lymph node-like" pattern and B-cell phenotype. A significant association also was observed between light chain restriction and low light chain expression ($P < .005$). **CONCLUSIONS:** The cytologic pattern of TLI in HT is quite representative of the clinical presentation and phenotypic cell type. Small light chain imbalances are not sustained by heavy chain Ig gene (IgH) rearrangements. FNA coupled with FC may contribute to making the distinction between florid TLI and non-Hodgkin lymphoma. **Cancer (Cancer Cytopathol) 2009;117:174–84. © 2009 American Cancer Society.**

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We thank Drs. Carmela Frangella, Antonino Iaccarino, and Maria Russo for technical assistance.

Received: August 25, 2008; **Revised:** October 22, 2008; **Accepted:** November 18, 2008

Published online: April 20, 2009 © 2009 American Cancer Society

DOI: 10.1002/cncy.20022, www.interscience.wiley.com

KEY WORDS: Hashimoto thyroiditis, fine-needle aspiration cytology, flow cytometry, polymerase chain reaction.

Hashimoto thyroiditis (HT) is the most frequent chronic autoimmune disease diagnosed in the Western world and develops as a result of the interaction between predisposing genetic factors and environmental triggers.[1-5] HT is characterized by the progressive loss of follicular cells and concomitant replacement of the thyroid by a lymphoid infiltrate (TLI) with the formation of germinal centers and fibrosis.[1,2] The gland may progressively shrink until completely atrophic, but in some patients or in some phases of the disease, there is symmetrical or asymmetrical enlargement of the gland with the formation of nodules. HT is also characterized by the presence of antiperoxidase and antithyroglobulin autoantibodies; an increase in serum thyroid-stimulating hormone (TSH) may herald incipient hypothyroidism. Other complications of HT are papillary carcinoma[6,7] and, rarely, non-Hodgkin lymphoma (NHL),[8] with mucosa-associated lymphoid tissue (MALT) lymphoma and diffuse large B-cell lymphoma (DLBCL) being the most frequently reported subtypes.[9] Fine-needle aspiration cytology (FNAC) is widely used in the diagnosis of thyroid nodules, including cases of HT with diffuse or nodular enlargement. Cytologic criteria for the diagnosis of HT are well-known,[10,11] as are the problems related to making the distinction between HT and thyroid lymphoma,[12] but to our knowledge relatively few studies to date have dealt with the cytologic and phenotypical characteristics of TLI.[13-15] Flow cytometry (FC) analysis applied to FNAC is a powerful tool in the study of hematologic neoplasms and κ/λ light chain assessment is an important criterion for discriminating between polyclonal reactive processes and monoclonal lymphomas.[16,17] Some studies performed on TLI using FC have revealed the existence of a κ/λ imbalance in HT lymphoid cells in a variable percentage of cases, without clinical evidence of NHL.[18,19] Moreover, a recent study using polymerase chain reaction (PCR) to amplify the variable diversity joining region (VDJ) of the heavy chain immunoglobulin (Ig) genes (IgH) has reported the presence of clonal bands in several HT cases without evidence of thyroid lymphoma during follow-up.[20] The objective of the current study was to classify the TLI in HT and compare the cytologic features with FC data, with a focus on the κ/λ light chain ratio and its molecular assessment.

MATERIALS AND METHODS

Patients

Between January 2005 and December 2007, 1456 patients with palpable thyroid nodules or diffuse swelling were admitted to the outpatient clinic of the Department of Pathology and Cytopathology for FNAC. Of these, 64 consecutive patients were diagnosed with or suspected to have HT because of the clinical, serologic, and/or ultrasound (US) presentation. Clinical signs and symptoms included a swollen thyroid gland with or without discrete nodules and, more rarely, mild fever. US examination had been previously performed or was available at the time of the final diagnosis of HT; the thyroid generally demonstrated poorly defined hypoechoic areas that occasionally were permeated by thin fibrous strands with a diffuse hypervascular pattern or diffuse glandular enlargement with scattered micronodules and a more hypoechoic echotexture than noted in normal thyroid parenchyma.

Thirty-four patients for whom a sufficient amount of cells could be collected to prepare the smear and to perform FC were enrolled in the current study. All the patients (all of whom were women ages 27-73 years) demonstrated palpable enlargement of the thyroid, had not received any previous pharmacologic treatment for thyroid pathologies, and presented with a US pattern that was consistent with HT. In fact, at US examination, the thyroid generally demonstrated the features reported earlier; moreover, in 28 of the selected cases, a discrete and palpable nodule with the same US features was found to be present. As a result, the US pattern was classified as nodular in 28 cases and diffuse in 6.

Serologic assessment was performed in all cases. The median values were as follows: TSH: 2.8 U/L (range, 0.9-16.1 U/L); free triiodothyronine (FT3): 3.4 pg/mL (range, 2.9-4.0 pg/mL); free thyroxine (FT4): 12.1 pg/mL (range, 8.0-14.3 pg/mL); thyroglobulin antibodies

(Tg-AB): 212 U/L (range, 86-1240 U/L); and thyroid peroxidase antibodies (TPO-AB): 89 (range, 48-612 U/L). Finally, FNAC confirmed the diagnosis of HT in all cases using the cytologic criteria reported below.

Although none of the patients were clinically suspected of having HT with lymphoma at the time of enrollment, they underwent special follow-up that included clinical, US, and serologic examinations performed every 6 months to evaluate clinical features, the size of the nodular areas and of the whole gland, and functional status. At the time of this report, this follow-up had been in progress for 11 to 35 months and no complications, including thyroid lymphoma, had been reported in any patients.

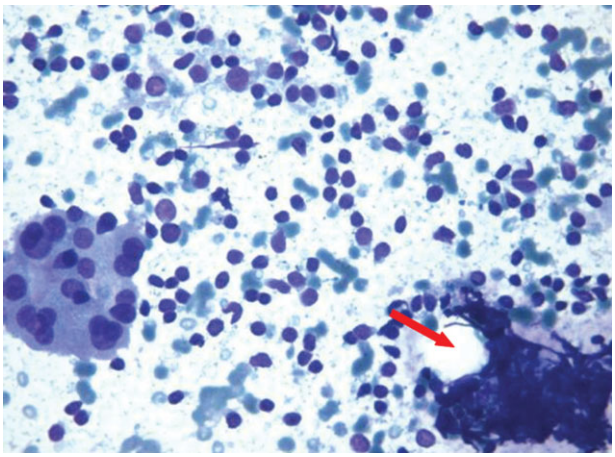

FIGURE 1. Cytologic features of the "lymphocytic" pattern of Hashimoto thyroiditis, in which small lymphocytes and occasional plasma cells are interspersed in the background. Note a group of Hurthle cells on the left and a lymphoid tangle on the right (arrow) (Diff-Quik, ×430).

FNAC

The diagnostic procedure and its related risks were first discussed with the patients, who were also informed that 1 or 2 supplementary FNAC specimens would be performed, before informed consent was obtained. FNAC was performed using palpation as previously described[7]; in the 2 or 3 passes that were performed, the needle was moved quickly back and forth in different directions, thereby ensuring that different areas of the swelling or nodule were sampled. In all cases, the first FNAC was used to prepare a traditional smear that was immediately stained with Diff-Quik and evaluated for adequacy; in the case of scanty cellularity, the procedure was immediately repeated. After the smear, the needle was washed with phosphate-buffered saline (PBS) solution and the contents carefully flushed out into in a tube; a second FNAC was then performed and the sample was flushed out with the same PBS solution and used for FC. In cases in which the smears were highly cellular with a large number of lymphocytes and follicular center cells, a third FNAC was performed and the sample flushed out with an RNA stabilization solution (RNAlater RNA stabilization reagent; QIAGEN, Hilden, Germany) and stored at −20°C.

Cytologic Classification

In keeping with the main cytologic criteria,[10,11] the diagnosis of HT was made on the basis of a lymphocytic infiltrate comprised of small lymphocytes, which were either isolated or intermingled with histiocytes, plasma cells, stretched lymphocytes, and germinal center lymphoid cells (eg, transforming lymphocytes, occasional centroblasts, and immunoblasts). Follicular cells and Hurthle cells were present in variable numbers; multinucleated giant cells were also present on occasion. All the cytologic diagnoses were confirmed by clinical, serologic, and US controls. All the smears were subsequently reviewed by 2 of the authors (P.Z. and I.C.), with a focus on the cytologic features of the TLI to quantify the different lymphoid components of the smears and classify the TLI accordingly. Two main cytologic patterns were recognized. In the first, the TLI was mainly comprised of small lymphocytes that were either isolated or intermingled with histiocytes, occasional plasma cells, and stretched lymphocytes. We therefore referred to this pattern as "lymphocytic" (Fig. 1). In the second cytologic pattern, a second cell population was observed in addition to that of small lymphocytes and plasma cells. This was comprised of larger lymphocytes and germinal center lymphoid cells that gave the smear a lymph node appearance; therefore, we called it a "lymph node-like" pattern (Fig. 2). Those smears that demonstrated both cytologic patterns were termed "mixed." Blind revision resulted in complete agreement and therefore all of the cases were reclassified as lymphocytic, lymph node-like, or mixed on the basis of their cytologic pattern.

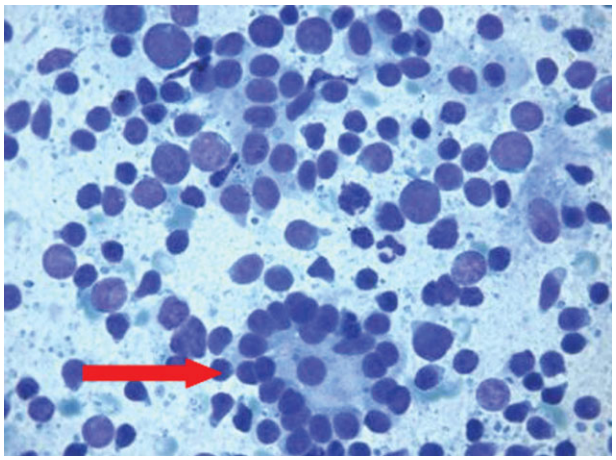

FIGURE 2. Cytologic features of the "lymph nodelike" pattern of Hashimoto thyroiditis. The smear demonstrates mainly small lymphocytes and a follicular center cell in different stages of maturation. A follicle structure of thyrocytes is present in the bottom of the figure (arrow) (Diff-Quik, ×430).

Flow Cytometry

FC smears were processed within 2 hours, washing twice by centrifugation for 4 minutes at 2500 revolutions per minute (rpm), removing the supernatant fluid, and adding 400 μL of PBS. The final suspension was divided between 3 or 4 tubes and incubated with the following combination of phycoerythrin (PE), perdin chlorophyll protein (PERCP), and fluorescein isothiocyanate (FITC) antibodies: CD3, CD4, CD8, CD5, CD10, CD19, and κ and λ light chains. Samples were then incubated for 15 minutes in the dark with 10 μL of antibody and, in the case of κ and λ light chains, in permeabilizing solution for 30 minutes in the dark at 4°C. All antibodies were purchased from BD Biosciences (San Jose, Calif). After incubation, red blood cells were lysed with ammonium chloride lysing solution (diluted to 10%) for 5 minutes followed by washing. When small fragments were still present, the suspension was filtered through 50-μm filters. Finally, an equal part of 1% paraformaldehyde was added to each tube for cell fixation. FC was then performed using a 3-color analysis technique on a Becton-Dickinson (San Jose, Calif FACS scan as previously described.[15] A gate of 5000 cells was considered sufficient for phenotypization and the expression of each antibody was quantified as a percentage of the whole gate. Light chain expression was calculated in each case by adding the percentage of κ and λ light chain expression and was evaluated as a percentage of the whole gate. We then classified each case as either having low light chain expression when the amount of expressing cells was <20% of the whole gate (≤20%) or as having high light chain expression when the amount of expressing cells was >20% of the whole gate (>20%). With regard to the κ/λ ratio, the nonlymphomatous polyclonal range is generally between 0.5 and 3.0,[16,17] whereas exceptional cases of reactive lymph nodes with κ/λ ratios as high as 7.0 have been reported.[21] Recently, a κ/λ ratio of >3.7 in reactive lymph nodes was reported[22] and imbalances of even >3.07 have been described in HT cases without evidence of thyroid lymphoma.[18,19] Therefore, we considered κ/λ ratios >4.0 to be imbalanced.

IgH, PCR, and Denaturing High-performance Liquid Chromatography Analyses

DNA extraction

In 4 of the 6 processed cases, sufficient high molecular weight DNA was extracted from the cells obtained by FNAC and stored in RNAlater using a commercially available kit (QIAamp DNA Mini Kit; QIAGEN) according to the manufacturer's instructions. Samples were centrifuged at 1300 rpm for 20 minutes to expel the RNAlater. After discharge of the supernatant fluid, 20 μL of proteinase K were added to each sample and incubation at 50°C for 3 hours was performed. After a second incubation at 70°C for 10 minutes in 200 μL of buffer AL (QIAGEN), 200 μL of ethanol (range, 96-100%) were added and the mixture was loaded onto a QIAamp Spin Column (QIAGEN) and centrifuged at 8000 rpm for 1 minute. The column was then washed with 500 μL of l buffer AW1 (QIAGEN) by centrifuging at 8000 rpm for 1 minute and 500 μL of l buffer AW2 (QIAGEN) by centrifuging at 14,000 rpm for 3 minutes. When all the residual ethanol had been removed, DNA was eluted with 70 mL of buffer AE (QIAGEN) by centrifuging at 8000 rpm for 1 minute.

IgH PCR

Using commercially available kits (B lymphomas; Nanogen, San Diego, Calif) according to the manufacturer's instructions, 2 PCR analyses were performed on the 4 HT cases plus 4 controls to amplify the VDJ region of the heavy chain Ig genes (IgH). Two of the 4 controls

were included in the kit (1 positive and 1 negative) and 2 proven lymphomas from our files were also used. This method uses a seminested PCR approach to increase the specificity and sensitivity of the reactions. In the first round, consensus primer FR2A, homologous to the framework 2 region of the variable heavy chain region, and consensus primer LJH, for joining the high chain region, were used. The second round of PCR was performed using the reverse primer VLJH, located just upstream of LJH, to amplify the first-round products. Cycle conditions for the first round were 94°C for 5 minutes followed by 30 cycles at 94°C for 45 seconds, 50°C for 45 seconds, and 72°C for 45 seconds and an additional 10-minute extension at 72°C. The same PCR temperature profile was used for the second round, but the cycle number was reduced to 20. Heteroduplex analysis was performed by loading 15 μL of PCR product onto a 4% agarose Tris-acetate-ethylenediamine tetraacetic acid (TAE) gel immediately after denaturing of the product at 94°C for 5 minutes and cooling at 4°C for 10 minutes. We then tested the DNA quality of the samples and the inhibition of PCR by amplifying the 5 exon of the p53 gene as a gene control (as described in the kit).

Denaturing high-performance liquid chromatography analysis

After heteroduplex induction, the clonality status of each product was evaluated by denaturing high-performance liquid chromatography (DHPLC) on a Transgenomic WaveTM System Model 3500HT (Transgenomic Inc, Omaha, Neb) on a high-resolution micropellicular matrix. Elution profiles were examined at 50°C (native DNA). Ten-μL samples were injected onto a preheated (50°C) C_{18} reversed-phase column with nonporous polystyrene-divinylbenzene particles (DNA Sep; Transgenomic). The injected sample was eluted with a linear acetonitrile gradient consisting of buffer A (0.1 mol/L of triethylammonium acetate [TEAA]) and buffer B (0.1 mol/L of TEAA and 250 mL/L of acetonitrile) with a 2% increase of buffer B occurring per minute. PCR products were separated with a flow rate of 0.9 mL/min. Retention time was measured online via ultraviolet absorbance at 254 nanometers (nm) in the elute. Resulting diagrams showed absorbance intensity in millivolts over the retention time in minutes (mV/min) after injection onto the column.

Statistical Analysis

Statistical analysis was performed to evaluate possibly significant associations between the clinical US presentation (nodular or diffuse) and the cytologic pattern (lymphocytic, lymph node-like, and mixed) using a chi-square test and, when necessary, a Fisher exact test. Statistical analysis was also performed to evaluate possible associations between cytologic patterns (lymphocytic or lymph node-like) and phenotypic cell type prevalence in the TLI (T lymphocytes [CD3 positive (+), CD5+], B lymphocytes [CD19+], and follicular center cells [CD10+]). Because these latter parameters had a non-normal distribution, possible associations were evaluated using a nonparametric test (Mann-Whitney *U* test). With regard to light chain expression, all cases were first classified according to the percentage of the expressing cells ($\leq$20% or $>$20%), and the chi-square test was then performed to evaluate the incidence of unbalanced cases in the 2 groups. All statistical tests were performed using SPSS software (SPSS Inc, Chicago, Ill).

RESULTS

Cytologic Patterns

Cytologic smears of HT demonstrated a variable number of normal follicular cells and Hurthle cells that were organized mainly in small loose groups, sometimes in follicular structures and sometimes isolated, with numerous dispersed lymphocytes in the background. The proportion of follicular cells, Hurthle cells, and lymphocytes varied, with a prevalence of 1 component in each case. Follicular cells were isolated or organized in small groups or follicular structures (Fig. 2). Nuclei were the same size as those of the small lymphocytes and even smaller than those of lymphoid follicular center cells. Therefore, they were not easily distinguishable from the latter (Fig. 2). Hurthle cells demonstrated the classically reported features,[11] being large with wide, grayish, occasionally granular, well-defined cytoplasm and 1 or 2 slightly eccentric nuclei (Fig. 1). These cells had variable anisonucleosis and sometimes quite large, atypical nuclei with dense chromatin and 1 or 2 evident nucleoli were present. The lymphoid infiltrate was comprised of a variable number of small lymphocytes with compact chromatin, plasma cells, macrophages with intermingled lymphocytes, and occasional

Table 1. Clinical, Cytologic, Flow Cytometry, and PCR Data for 34 Cases of Hashimoto Thyroiditis

Case No.	Age, Years/ Gender	US Pattern	Cytologic Pattern	CD3+, CD5+, %	CD19+, %	CD10+, %	CD10+, CD19+, %	k+λ, %	κ/λ	PCR
1	28/Woman	Nodular	Lymph node-like	21	5	40	0	11	4.50	NS
2	54/Woman	Diffuse	Lymphocytic	54	10	35	0	6	2.00	NP
3	48/Woman	Nodular	Mixed	16	13	45	0	0	0.00	NP
4	44/Woman	Nodular	Lymphocytic	60	30	0	0	28	2.50	NP
5	47/Woman	Nodular	Lymphocytic	66	10	0	0	4	1.00	NP
6	47/Woman	Diffuse	Lymphocytic	28	10	40	0	0	0.00	NP
7	34/Woman	Nodular	Mixed	30	18	12	12	23	1.09	NP
8	27/Woman	Nodular	Lymph node-like	61	24	16	13	24	1.40	NP
9	46/Woman	Nodular	Mixed	55	37	19	19	28	1.15	NP
10	54/Woman	Nodular	Lymph node-like	54	35	8	5	35	2.18	NP
11	47/Woman	Nodular	Lymph node-like	50	32	22	20	21	4.25	Neg
12	41/Woman	Nodular	Lymphocytic	72	20	0	0	28	1.54	NP
13	56/Woman	Diffuse	Lymphocytic	74	10	0	0	10	4.00	Neg
14	52/Woman	Nodular	Mixed	48	21	0	0	28	1.00	NP
15	23/Woman	Diffuse	Lymphocytic	66	29	20	18	16	1.00	NP
16	50/Woman	Nodular	Lymphocyte	65	10	0	0	23	1.50	NP
17	51/Woman	Nodular	Lymph node-like	18	21	11	10	10	1.50	NP
18	54/Woman	Nodular	Mixed	54	36	0	0	32	1.13	NP
19	29/Woman	Nodular	Mixed	59	22	19	10	0	0.00	NP
20	46/Woman	Nodular	Lymphocytic	30	43	20	18	25	3.16	NP
21	54/Woman	Nodular	Lymph node-like	60	36	6	5	30	1.50	NP
22	55/Woman	Diffuse	Lymphocytic	67	8	0	0	0	0.00	NP
23	73/Woman	Nodular	Lymph node-like	20	43	10	10	31	4.16	Neg
24	58/Woman	Nodular	Lymphocytic	70	20	0	0	7	1.33	NP
25	58/Woman	Nodular	Lymph node-like	38	30	0	0	11	1.20	NP
26	50/Woman	Nodular	Lymph node-like	50	30	0	0	27	1.07	NP
27	47/Woman	Diffuse	Mixed	60	25	5	0	20	1.22	NP
28	42/Woman	Nodular	Lymph node-like	34	16	0	0	7	1.33	NP
29	25/Woman	Nodular	Lymphocytic	47	22	0	0	22	1.2	NP
30	46/Woman	Nodular	Lymph node-like	45	37	25	25	35	1.69	NP
31	34/Woman	Nodular	Lymph node-like	40	33	12	12	16	3.00	NS
32	55/Woman	Nodular	Mixed	36	16	10	7	15	1.14	NP
33	38/Woman	Nodular	Lymph node-like	39	52	17	15	44	1.31	NP
34	38/Woman	Nodular	Lymph node-like	12	10	50	0	12	5.00	Neg

PCR indicates polymerase chain reaction; US, ultrasound; +, positive; k+λ %, percentage of light chain-producing cells within the whole gate; κ/λ, light chain ratio; NS, not sufficient DNA; NP, not performed; Neg, negative for clonality by PCR for immunoglobulin H gene rearrangement.

bundles of crushed lymphocyte nuclei, referred to as "lymphoid tangles" (Fig. 1). In some cases, there were also follicular center cells in different stages of maturation, ranging from medium to large, and immunoblasts, giving the smear a lymph node-like appearance (Fig. 2). The entire series was reviewed by 2 of the authors and each case was classified, according to the cytologic pattern, as lymphocytic (12 cases), lymph node-like (14 cases), or mixed (8 cases) (Table 1).

FC Findings

FC demonstrated 2 main immunophenotypical cell populations of CD3+, CD5+, and CD19+ lymphocytes, ranging between 12% and 74% and 8% and 52%, respectively. There was a prevalence of CD3+, CD5+ (Fig. 3) in 30 of 34 cases and of CD19+ in 4 of 34 cases (Fig. 3). CD3+, CD5+ lymphocytes were present in all cases; in the absence of a significant number of CD19+ cells, (<20% of the gated cells), we classified these cases as being of the T-cell phenotype. Conversely, in 22 cases, there was a significant presence of CD19+ (≥20%), and we determined these cases to be of the B-cell phenotype (Fig. 4). CD10+ cells were observed in 21 of 34 cases, and in 14 cases were associated with CD19+ with a variable percentage of coexpression (Fig. 5). In 5 cases, CD10+ cells represented a separate cluster in the forward scatter histogram with CD19+ cells identified in another cluster if present. Light chains were expressed in 30 of 34 cases; in 13 cases, light chains were expressed in <20%

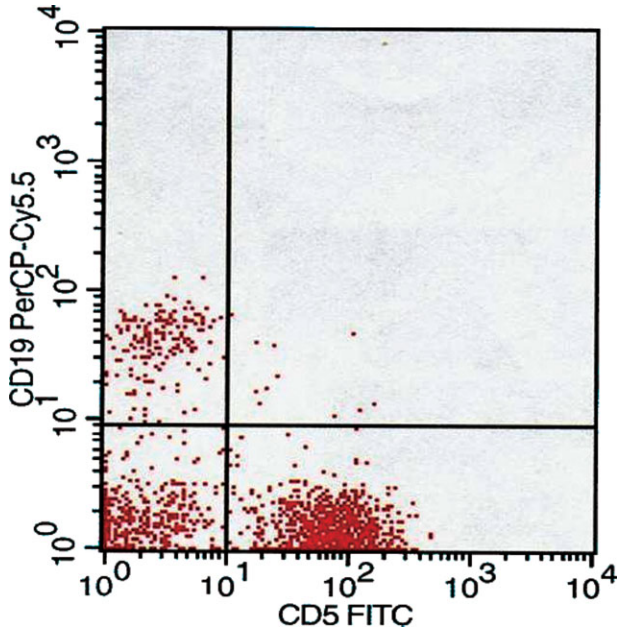

FIGURE 3. Flow cytometry of "lymphocytic" Hashimoto thyroiditis. There was predominant CD5 expression in the lower right quadrant and low expression of CD19 (<20% of the gate) in the upper right quadrant. PerCP indicates perdin chlorophyll protein; FITC, fluorescein isothiocyanate.

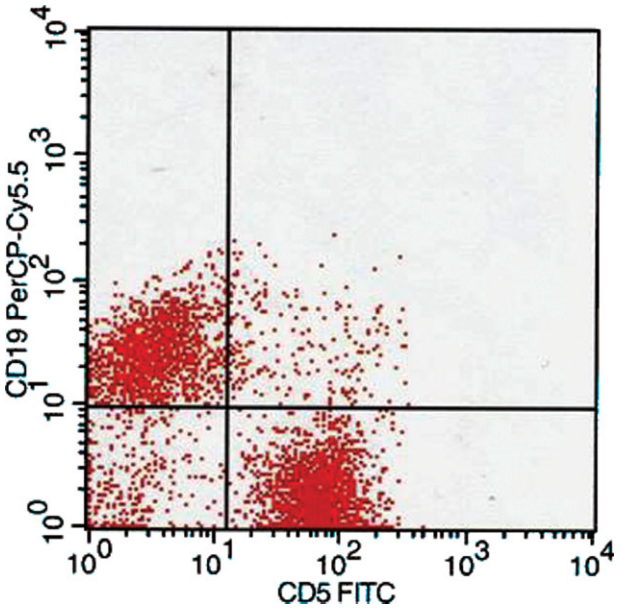

FIGURE 4. Flow cytometry of "lymph node like" Hashimoto thyroiditis. CD5 (lower right quadrant) and CD19 (upper left quadrant)-positive cells (>20% of the entire gate) are shown. PerCP indicates perdin chlorophyll protein; FITC, fluorescein isothiocyanate.

of the gated cells and in >20% in 17 cases. With regard to the κ/λ ratio, 5 of the 34 cases demonstrated a κ/λ light chain ratio of >3.7 (≥4.0) (Fig. 6); in the remaining cases, there was a balanced ratio (≤3.10 and ≥1.00) (Figs. 5 and 6). Finally, when comparing the κ/λ unbalanced cases with the quantitative percentage light chain-expressing cells, 3 of these cases were found to be low producing (≤20%) (Cases 1, 13, and 34) and 2 were found to be high producing (>20%) (Cases 11 and 23). Clinical, cytologic, and FC data are summarized in Table 1.

PCR Findings

Heteroduplex analysis of the 2 lymphoma controls and the positive control supplied with the kit demonstrated 1 or 2 thin clear bands within the expected size range of 240 to 260 base pairs (bp), confirming monoclonality. Analysis of the 4 thyroiditis cases and the negative control supplied with the kit resulted in rough smears (strong diffuse bands) in the gel (Fig. 7). After heteroduplex and homoduplex formation, all samples analyzed demonstrated the expected band of 274 bp in the p53 gene control in the gel (data not shown).

DHPLC Analysis

The heteroduplex of the 4 HT cases demonstrated Gaussian curves; no detectable single peaks were observed in the negative control. In contrast, a clonal PCR product forming 3 distinct homoduplex peaks was observed in the 2 positive controls as well as in the positive control, provided by the kit, containing monoclonal IgH gene rearrangements (Fig. 8).

Statistical Findings

Statistical analysis demonstrated significant associations between a diffuse clinical presentation and the lymphocytic cytologic pattern and between a nodular clinical presentation and the lymph node-like cytologic pattern ($P < .005$). Statistically significant associations were also found between the lymphocytic cytologic pattern and T-cell phenotype and between the lymph node-like cytologic pattern and B-cell phenotype ($P < .05$). With regard to light chain expression, a significant association was observed between light chain restriction and low light chain expression ($P < .005$).

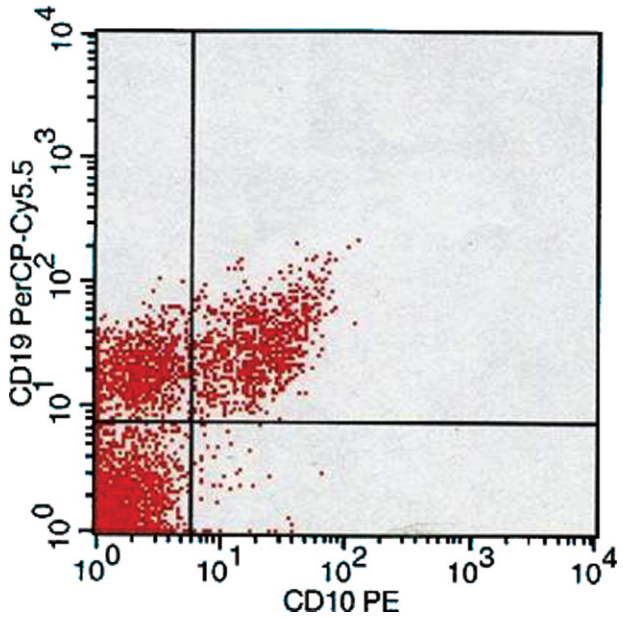

FIGURE 5. Flow cytometry of "lymph node like" Hashimoto thyroiditis. Two clusters of CD19 (upper left quadrant) and CD19/CD10 coexpression in the upper right quadrant assessing the "follicular" pattern of the B cell. PerCP indicates perdin chlorophyll protein; PE, phycoerythrin.

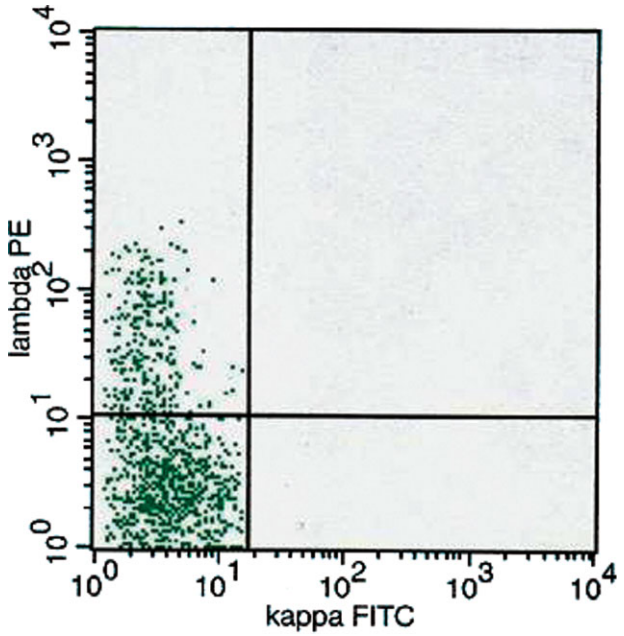

FIGURE 6. Flow cytometry of the κ/λ light chain ratio in a "microclonal" Hashimoto thyroiditis sample. Nearly all of the cells expressed the κ light chain but producing cells were <20% of the whole cell gate. PE indicates phycoerythrin; FITC, fluorescein isothiocyanate.

DISCUSSION

HT is characterized by progressive loss of thyroid follicular cells and concomitant replacement of the gland by lymphocytes, with the formation of germinal centers and fibrosis.[1] Apoptosis is generally agreed to be the main pathogenetic mechanism of follicular cell destruction; predominantly Th1-mediated immune activity may promote apoptosis of thyroid follicular cells, leading to thyroid cell destruction and HT.[23,24] In fact, overexpression of FAS and FAS-L by follicular cells and cytokine secretion by T lymphocytes induce apoptosis, although a direct cytotoxic mechanism is almost certainly possible.[25] The gland is colonized by B lymphocytes, which participate in the autoimmune process by inducing the formation of autoantibodies and forming lymphoid follicles. B lymphocytes also constitute the basis for the possible onset of thyroid lymphoma. With regard to the temporal evaluation of the appearance of these 2 lymphoid cell populations, it is conceivable that T lymphocytes are the first to colonize the gland, followed by B lymphocytes with germinal center formation as the disease becomes completely established.[1,2,26] Therefore, the TLI is responsible for symmetrical or asymmetrical enlargement as well as for progressive atrophy and reduction in the size of the gland. The current study focused on the phenotypic and quantitative features of the TLI using FC and compared the TLI with the clinical presentation and cytologic pattern. We observed significant associations between clinical symmetrical enlargement and the lymphocytic cytologic pattern and between clinical nodular presentation and the lymph node-like cytologic pattern. With regard to the phenotypic features of TLI, significant associations were found between the lymphocytic cytologic pattern and CD3+, CD5+ lymphocyte prevalence and between the lymph node-like pattern and CD19+ and CD10+/- phenotype; therefore, these results suggest that the cytologic features of TLI may be consistent with its phenotype. As reported earlier, it is not possible to determine a possible temporal consequentiality or to exclude the possible coexistence of different cell types in different areas of the gland; moreover, follicular cells and Hurthle cells also may contribute to symmetrical or asymmetrical enlargement. These limitations notwithstanding, the data from the current study suggest that a lymph node-like infiltrate with the presence of B lymphocytes with the formation of lymphoid

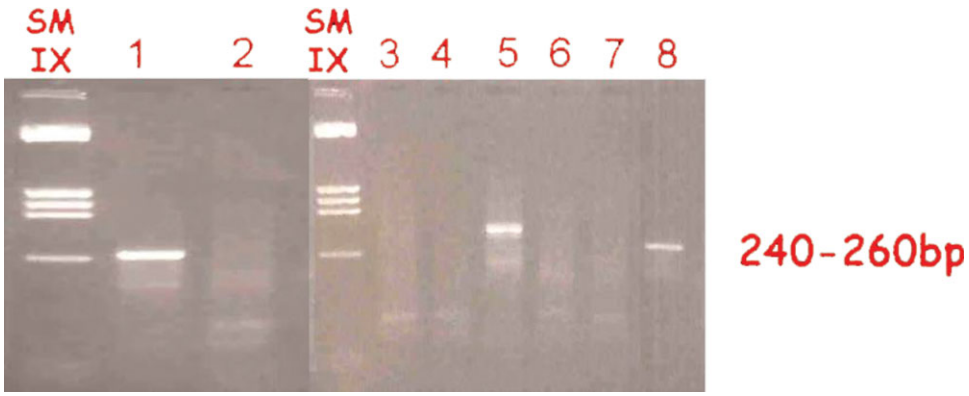

FIGURE 7. Heteroduplex analysis of monoclonal (line 1-5-8) and polyclonal (line 2-3-4-6-7) of immunoglobulin (Ig) H rearrangements obtained by seminested polymerase chain reaction. Lane 1 indicates positive control from the kit; Lanes 2-3-4-6, Hashimoto thyroiditis; Lane 5-8, lymphomas; and Lane 7, negative control from the kit. SMIX, DNA molecular weight marker IX; bp, base pair.

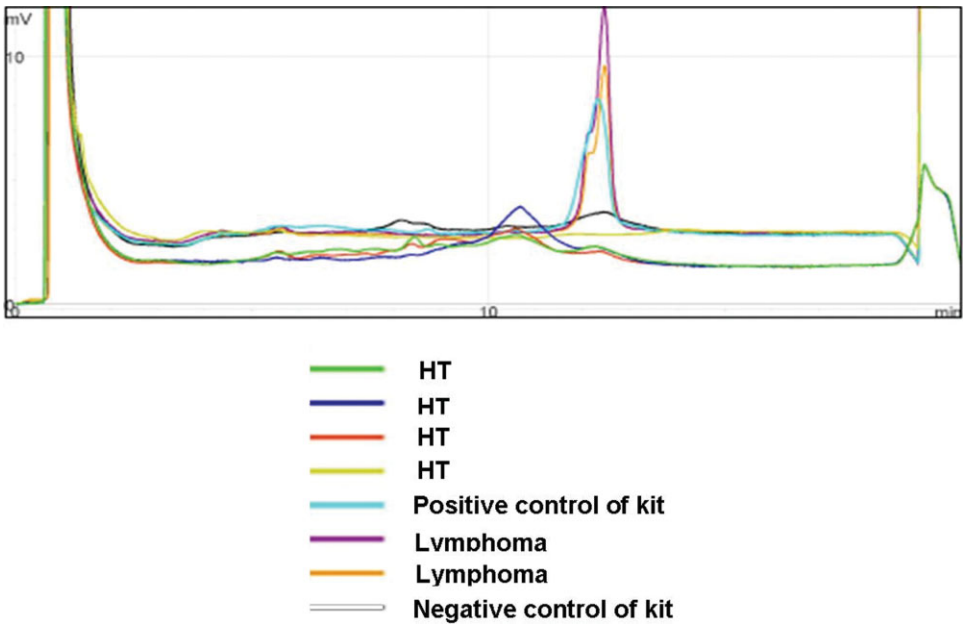

FIGURE 8. Denaturing high-performance liquid chromatography analysis of monoclonal (orange and violet peaks, lymphoma; light blue peak, positive control) and polyclonal (blue Gaussian curve) products of immunoglobulin (Ig) H rearrangements obtained by seminested polymerase chain reaction. mV indicates millivolts; HT, Hashimoto thyroiditis.

follicles may contribute to asymmetrical and hence nodular enlargement of the gland.

Light chain evaluation is generally considered to be important in the phenotypic discrimination between polyclonal and monoclonal lymphoid processes. The normal range in benign reactive processes is considered to be from 0.5 to 3.0,[17] although some reports have cited significantly higher κ/λ ratios in reactive lymph nodes during various inflammatory processes.[21] This phenomenon has also been described in HT.[18] A recent study[19] reported a κ/λ ratio of >3.7 in 67% of cases studied, without IgH rearrangement or any evidence of lymphoma noted during follow-up. As reported earlier, we also observed a κ/λ ratio of >4.0 in 5 of the cases in the current study. In 4 of these 5 cases, molecular analysis of the IgH locus demonstrated the absence of rearrangements, showing diffuse

bands in PCR analysis and Gaussian curves, instead of peaks, with DHPLC. Moreover, although follow-up was not yet extensive at the time of this report, none of our cases had developed NHL at the time of last follow-up. These data further confirm the existence of B-cell populations with light chain restriction in a variable number of HT cases, although the percentage is quite variable.[18,19] In fact, in the studies currently available in the literature, the percentage ranges from 30% to 67%[18,19]; this might be related to different technical procedures. It also has been observed that if one focuses on the CD10+ cells, the percentage is higher; in fact, Chen et al[19] observed a κ/λ ratio of >3.7 in 67% of CD10+ and 10% of CD10- cases; these data are closer to the results of the current study. We did not concentrate on the light chain evaluation of CD10+ cells alone because we were interested in the entire B-cell population; moreover, primary follicular (CD10+) lymphomas of the thyroid are quite rare, whereas MALT and DLBCL are the most frequently observed histotypes. As reported earlier, in addition to the κ/λ ratio, we also evaluated the percentage of the cell population expressing light chains (≤20% and >20%) and observed that 13 of 34 cases, including those found to be unbalanced, were low producing (≤20%) ($P < .005$). In other studies,[18,19] this quantitative aspect of light chain production was not reported, although the same authors stated that the B cell percentage in the TLI of their "clonal" cases represented a minority of the cell population.[19] We would therefore call this phenomenon "microclonality," and it may represent an additional criterion for the differentiation of HT from lymphomatous processes. A recent study also demonstrated the presence of clonal bands in 3 of 10 HT cases using PCR to amplify the IgH gene VDJ region, although these clonal bands could not be reproduced from other blocks or from deeper sections of the same block.[20] Moreover, none of the corresponding patients developed malignant lymphoma during follow-up.[20] At the same time, a study performed on a series of cases with thyroid lymphoma and coexisting thyroiditis demonstrated sequence similarities between the clonal bands in HT and the subsequent thyroid lymphoma within the same histologic samples.[12] These data further support the concept that thyroid lymphoma may evolve from HT; nonetheless, the presence of "microclones" identified by FC do not appear to be prelymphomatous conditions or "lymphomas in situ" as described in lymph nodes.[27] In conclusion, the cytologic pattern of the TLI in HT is quite representative of the clinical presentation and phenotypic cell type prevalence. With regard to the κ/λ ratio, quantitatively small imbalances detected by FC are not sustained by heavy chain Ig gene (IgH) rearrangements and appear to be a reactive phenomenon rather than a prelymphomatous condition. Finally, FNAC may contribute, in a specific clinicocytologic setting, to the distinction between florid TLI and NHL, although strict follow-up, at least, appears appropriate considering the to our knowledge limited experiences available.

Conflict of Interest Disclosures

The authors made no disclosures.

References

1. Weetman AP. Autoimmune thyroid disease: propagation and progression. *Eur J Endocrinol*. 2003;148:1-9.
2. Larsen PR, Ingbar SH. The thyroid gland. In: Wilson JD, Foster DW, eds. Williams Textbook of Endocrinology. 8th ed. Philadelphia: W.B. Saunders; 1992:357-487.
3. Dong Z, Takakuwa T, Takayama H, et al. Fas and Fas ligand gene mutations in Hashimoto's thyroiditis. *Lab Invest*. 2002;82:1611-1616.
4. Tomer Y, Davies TF. Searching for the autoimmune thyroid disease susceptibility genes: from gene mapping to gene function. *Endocr Rev*. 2003;24:694-717.
5. Stuck BJ, Pani MA, Besrour F, et al. No association of 2 Fas gene polymorphisms with Hashimoto's thyroiditis and Graves' disease. *Eur J Endocrinol*. 2003;149:393-396.
6. Liu LH, Bakhos R, Wojcik EM. Concomitant papillary thyroid carcinoma and Hashimoto's thyroiditis. *Semin Diagn Pathol*. 2001;18:99-103.
7. Zeppa P, Benincasa G, Lucariello A, Palombini L. Association of different pathologic processes of the thyroid gland in fine needle aspiration samples. *Acta Cytol*. 2001;45:347-352.
8. Kossev P, Livolsi V. Lymphoid lesions of the thyroid: review in light of the revised European-American lymphoma classification and upcoming World Health Organization classification. *Thyroid*. 1999;9:1273-1280.
9. Thieblemont C, Mayer A, Dumontet C, et al. Primary thyroid lymphoma is a heterogeneous disease. *J Clin Endocrinol Metab*. 2002;87:105-111.
10. Buley ID The thyroid gland. In: Grey W, McKee GT, eds. Diagnostic Cytopathology. 2nd ed. New York: Elsevier Science; 2003:584-587.
11. Kini SR. Guides to Clinical Aspiration Biopsy: Thyroid. 2nd ed. New York: Igaku-Shoin; 1996.
12. Moshynska OV, Saxena A. Clonal relationship between Hashimoto thyroiditis and thyroid lymphoma. *J Clin Pathol*. 2008;61:438-444.

13. Tani E, Skoog L. Fine needle aspiration cytology and immunocytochemistry in the diagnosis of lymphoid lesions of the thyroid gland. *Acta Cytol*. 1989;33:48-52.
14. Guarda LA, Baskin HJ. Inflammatory and lymphoid lesions of the thyroid gland. Cytopathology by fine-needle aspiration. *Am J Clin Pathol*. 1987;87:14-22.
15. Zeppa P, Marino G, Lepore M, et al. Flow cytometry phenotypization of thyroidal lymphoid infiltrate and functional status in Hashimoto's thyroiditis. *Anal Quant Cytol Histol*. 2006;28:148-516.
16. Kaleem Z. Flow cytometric analysis of lymphomas: current status and usefulness. *Arch Pathol Lab Med*. 2006;130: 1850-1858.
17. Geary WA, Frierson HF, Innes DJ, Normansell DE. Quantitative criteria for clonality in the diagnosis of B-cell non-Hodgkin's lymphoma by flow cytometry. *Mod Pathol*. 1993;6:155-161.
18. Matsubayashi S, Tamai H, Morita T, et al. Hashimoto's thyroiditis manifesting monoclonal lymphocytic infiltration. *Clin Exp Immunol*. 1990;79:170-174.
19. Chen HI, Akpolat I, Mody D, et al. Restricted kappa/lambda light chain ratio by flow cytometry in germinal center B cells in Hashimoto thyroiditis. *Am J Clin Pathol*. 2006;125:42-48.
20. Saxena A, Alport EC, Moshynska O, Kanthan R, Boctor MA. Clonal B cell populations in a minority of patients with Hashimoto's thyroiditis. *J Clin Pathol*. 2004;57:1258-1263.
21. Kussick SJ, Kalnoski M, Braziel RM, Wood BL. Prominent clonal B-cell populations identified by flow cytometry in histologically reactive lymphoid proliferations. *Am J Clin Pathol*. 2004;121:464-472.
22. Reichard KK, McKenna RW, Kroft SH. Comparative analysis of light chain expression in germinal center cells and mantle cells of reactive lymphoid tissues. A 4-color flow cytometric study. *Am J Clin Pathol*. 2003;119:130-136.
23. Giordano C, Richiusa P, Bagnasco M, et al. Differential regulation of Fas-mediated apoptosis in both thyrocyte and lymphocyte cellular compartments correlates with opposite phenotypic. *Thyroid*. 2001;11:233-244.
24. Mirakian R, Nye K, Palazzo FF, Goode AW, Hammond LJ. Methods for detecting apoptosis in thyroid diseases. *J Immunol Methods*. 2002;265:161-175.
25. Watanabe M, Yamamoto N, Maruoka H, et al. Independent involvement of CD8+ CD25+ cells and thyroid autoantibodies in disease severity of Hashimoto's disease. *Thyroid*. 2002;12:801-808.
26. Quaratino S, Badami E, Pang YY, et al. Degenerate self-reactive human T-cell receptor causes spontaneous autoimmune disease in mice. *Nat Med*. 2004;10:920-926.
27. Cong P, Raffeld M, Teruya-Feldstein J, Sorbara L, Pittaluga S, Jaffe ES. In situ localization of follicular lymphoma: description and analysis by laser capture microdissection. *Blood*. 2002;99:3376-3382.