The differences in T and B cell subsets in thyroid of children with Graves' disease and Hashimoto's thyroiditis

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Background: **The differences between Graves' disease (GD) and Hashimoto's thyroiditis (HT) suggest that changes in the subsets of T cells may have an influence on the course of these reactions.**

Methods: **This study included 90 children: 30 with GD, 30 with HT, and 30 healthy children as controls. After thyroidectomy, standard histological examinations and immunohistochemical reactions were performed in paraffin specimens with monoclonal antibodies against T cell markers CD3, CD4, CD8 as well as against CD79 alpha B cells. Ultrathin sections were examined under a transmission electron microscope.**

Results: **Autoimmune reaction in GD consisted of an increased number of CD4+ T cells (3.17±4.27%) and plasma cells (22.89±8.61%) producing thyroid-stimulating hormone-receptors and stimulating thyrocytes to activity. The number of CD8+ T cells was increased in children with HT (20.54±0.68%) as compared with the controls (0.65±0.30%). The autoimmune reaction in the HT children showed antibody dependent cytotoxicity with a low number of CD4+ T cells and an increased number of CD8+ T cells in the thyroid tissue in comparison with that in the GD children and the controls. Plasma cells (31.65±9.11%) in this situation produced the antibodies involved in cytotoxic reactions against thyrocytes.**

Conclusions: **Graves' disease is characterized by the increased number of CD4+ T cells and CD8+ T cells. Hashimoto's thyroiditis is characterized by the low number of CD4+ T cells and increased number of CD8+ T cells. CD8+ T cells have cytotoxic properties only in Hashimoto's thyroiditis.**

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Original article

Introduction

Autoimmune thyroid disorders (AITDs) are caused by a reaction between thyroid self-antigens and the body's immune system.[1,2] The disease-specific antibodies can be produced against thyroid-stimulating hormone (TSH)-receptors, thyroperoxidase (TPO Ab) and thyroglobluline (TG Ab).[1,2]

The pathogenic agents of Graves' disease (GD) are antibodies against the TSH receptors (TRAb). The receptor-stimulating antibodies react with the TSH-receptors and stimulate production of thyroid hormones as well as growth and proliferation of thyrocytes.[2,3] Other antibodies can block connections between TSH and the receptors.[4,5] The third group of antibodies, the TSH-binding inhibitory immunoglobulin, bind to the thyrotropine receptor but do not inhibit or stimulate their function.[5,6] Probably, each patient with GD exhibits a mixture of antibodies in different proportions. Stimulatory antibodies are the most predominant.

Hashimoto's thyroiditis (HT) is at the opposite pole of autoimmune reaction. Autoimmune Hashimoto's thyroiditis is characterized by a painless, enlarged, normal or decreased thyroid, exhibiting lymphoid cell infiltrations, fibrosis, and atrophic changes in histopathologic investigations. Increased levels of thyroglobuline antibodies (TGAb) and thyroperoxidase antibodies (TPOAb) are detected in the serum of patients with HT. Antibody-dependent cellular cytotoxicity (ADCC) and T cytotoxic cells, natural killer and killer cells or T regulatory cells (Treg), are supposed to play an important role in this process.[7-9] Some investigations[10-16] found a possible T cell-mediated transfer of autoimmune processes in the thyroid both in animals and humans, for example, as a result of bone marrow transplantation.

Most often, autoimmune reactions are assessed indirectly by investigations of the levels of thyroid autoantibodies, cytokines and chemokines in serum, and subsets of lymphocytes in peripheral blood samples. Such experiments are conducted on animal models or cell cultures.

The aim of the present study was to directly evaluate the immune reaction in the parenchyma of the thyroid gland of young patients with autoimmune thyroid diseases, GD and HT, and to explore the differences between the two diseases.

Methods

Patients

The study involved 90 children: 30 children affected with GD, 30 with HT, and 30 as controls. The children were treated in the Department of Pediatric Endocrinology and Neurology in Lublin and in the Pediatric Department in Rzeszow in the period of 1994-2007 and were operated on in the Surgery Department of the Voivodship Hospital in Lublin and the Voivoidship Hospital in Rzeszow. The investigation was accepted by the local Ethical Committee at the Medical University of Lublin. Parents and patients signed an informed consent before these investigations.

The control group consisted of 30 children aged 6-19 years who had died from accidents and other non-autoimmune diseases; thyroid specimens were taken during autopsy (n=25). Part of the specimens were taken during a resection of thyroglossal cysts or during surgery for parathyroid glands (n=5). These were fragments of routinely sampled tissue specimens for standard pathologic investigations; the thyroids exhibited a normal structure. All the children were euthyroid (Table 1).

All the patients received a physical examination to assess the goitre and clinical signs and symptoms of thyroid disorders. The levels of thyroid-stimulating hormone (TSH), free thyroxine (fT4) and total triiodothyronine (TT3) were assayed by MEIA (Abbott Kit, Langford, Ireland). The levels of TSH receptor antibodies were measured by RIA (TRAB assay BRAHMS Diagnostica GmbH, Berlin, Germany). Thyroperoxidase (TPO) and thyroglobulin (TG) antibodies were assayed by LIA (Lumitest BRAHMS Diagnostica GmbH, Berlin, Germany).

GD was diagnosed when patients had symptoms of thyrotoxicosis, i.e. goitre, tachycardia, sleeplessness, anxiety, high diastolic/systolic blood pressure, and an increase in free T4 and TT3, and a decrease in TSH. The levels of antibodies against the TSH receptor (TRAb) were increased. The patients were treated with methimazole in initial doses of 0.5-0.9 mg/kg b.w./day for 4-6 weeks and afterwards they received maintenance doses of c.a.0.1 mg/kg b.w./day for 18-24 months in combination with a low dose of L-thyroxine (25 µg/day). Patients with GD with early relapse of hyperthyroidism that necessitated thyroidectomy within 18-36 months were qualified for the investigation (Table 1).

HT was recognized in patients with parenchymal or nodular goitre in the phase of hypothyroidism, rarely in hyperthyroidism (hashitoxicosis). A non-homogenous structure of the thyroid was observed by ultrasonography. The levels of TPOAb and TGAb were increased, but the levels of TRAb were within normal ranges. Mononuclear lymphatic infiltrations were observed histopathologically in the thyroid parenchyma. Before surgery, these patients were usually treated with L-thyroxine 25-100 µg/day; they were operated on because of the large size of the goitre, which exerted pressure on other neck structures (Table 1).

Immunohistochemical investigations

Immunohistochemical reactions were performed in paraffin specimens with monoclonal antibodies against T-cell markers CD3, CD8 as well as against B-cells-CD79 alpha antibodies (DakoCytomation Denmark) and CD4 monoclonal antibodies (Novocastra, Germany). Thyroid specimens were incubated with the primary antibody, followed by with dextran marked with horseradish peroxidase (Dakocytomation EnVision+Peroxidase kit) and with the chromogene of DAB horseradish. When the immunohistochemical reaction was finished, cell nuclei were stained with Mayer's hematoxylin. The specimens were dehydrated

Table 1. Characteristics of the examined patients before treatment.

	No.	Age, y	TSH, mIU/L	fT4, ng/dL	TPO Ab, IU/L	TG Ab, IU/L	TRAb, IU/L	Pathomorphological diagnosis (hematoxilin-eosin staining)
Graves' disease	30	5-19	0.001-0.005	3.3-5.1	21-6663	25-13351	7-462	Graves' disease
Hashimoto's thyroiditis	30	8-19	0.600-98.800	0.1-2.3	132-9856	128-14567	0-0.99	Hashimoto's thyroiditis
Control group	30	6-19	0.270-4.000	not available	not available	not available	not available	Healthy thyroid
Normal ranges			0.270-4.200	0.8-2.3	<34	<115	<1	

TSH: thyroid-stimulating hormone; fT4: free thyroxine; TPO Ab: thyroperoxidase antibodies; TG Ab: thyroglobulin antibodies; TRAb: antibodies agains TSH-receptor.

in alcohol, exposed to xylene, and embedded in Canada balsam. Control examinations without monoclonal antibodies were performed for each specimen to exclude nonspecific reactions.

The specimens were estimated under an Axiostar plus microscope. Lymphocytes were counted in a Sony Colour Camera ExwaveHAD and the lymphocyte subsets were analysed using MultiScan5. We determined the number of lymphocyte subpopulations in the thyroid tissue by counting lymphocytes marked with CD3+, CD4+, CD8+, CD79 alpha + monoclonal antibodies in every 1000 cells as present in 10 vision fields of the microscope and by estimating their percentage content.

Ultrastructural investigations

Specimens for ultrastructural investigations were obtained during thyroidectomy (n=74) or from paraffin dewaxed preparations (n=16). Small segments of the thyroids were cut into 0.5 mm³ pieces and fixed in 4% glutaraldehyde in 0.1 mol/L cacodylate buffer, pH 7.4 for 24 hours in 4ºC, post fixed in 2% OsO_4 in the same buffer for 1 hour in room temperature, dehydrated in a graded series (up to 100%) of ethanol and embedded in 812 Epon. They were then polymerized at 60 ºC. The Epon blocks were cut with the RMC MT-7 ultramicrotome, USA. The ultrathin sections were contrasted with uranyl acetate and lead citrate and examined under an EM 900 Zeiss Germany Electron Microscope.

The results were expressed as mean±SD. The percentage of lymphocytes expressing receptors to monoclonal antibodies was compared using the Kruskal-Wallis U test. T value was considered statistically significant at P<0.05. Mathematical and statistical calculations were performed with Statistica 7.0.

Results

T cells

The increased percentage of CD3+cells in the vision fields was observed in GD. T cells were particularly accumulated in the lymphatic infiltrations. They were present in the parenchyma among the thyroid follicles; the thyrocytes in this area were undamaged and very active (columnar, with big nuclei, well developed microvilli and rough endoplasmic reticulum). More T cells were observed in HT both in the parenchyma and in the lymphatic infiltrations. In HT, damaged thyroid follicles with apoptotic thyrocytes (pyknotic nuclei, condensed cytoplasm with enlarged mitochondria and endoplasmic reticulum cisterns) were visible in this area.

CD4+ T cells occurred sporadically in the thyroids of the control group. In GD, the number of CD4+ T cells was the largest, especially in mononuclear infiltrates. The lowest percentage of CD4+ T cells was observed in HT in the parenchyma and in infiltrates. Compared to the control group, the number of CD4+ T cells in thyroid infiltrates in HT was not statistically different but it was significantly decreased in the interstitium (Table 2).

The subsets of CD8+ T cells were present in a low number of thyroids in patients without a thyroid disease. In GD, CD8+ T cells were more numerous in the mononuclear infiltrations than in the rest of the thyroid parenchyma. The greatest number of CD8+ T cells was observed in HT (Table 2). Observations under a light microscope demonstrated that T suppressor/cytotoxic cells were accumulated at the sites of destruction of thyroid follicles in HT. These sites were surrounded by connective tissue fibres and fibroblasts. No damage of neighbouring thyrocytes was detected in GD or in the control group.

Active B lymphocytes-plasma cells

A low number of active B cells CD79 alfa+, i.e. antibody-producing plasma cells, were visible in the thyroid specimens from the control group. An increased percentage of B cells CD79 alfa+ was observed in GD, especially in the mononuclear infiltrates. The number of plasma cells in the interstitium was slightly increased,

Table 2. Subsets of T cells in Graves' disease (GD) and in Hashimoto's thyroiditis (HT)

		CD3+ T cells	P^*	CD4+ T cells	P^*	CD8+ T cells	P^*
Mean	GD	17.79±8.75%	0.000	3.17±4.27%	0.000	6.86±3.46%	0.000
	HT	30.38±10.08%		0.93±9.90%		20.54±0.68%	
Parenchyma	GD	5.07±6.09%	0.000	1.17±1.94%	0.000	2.49±2.85%	0.093
	HT	22.88±14.02%		0.07±0.12%		17.32±14.01%	
Lymphatic infiltrations	GD	30.39±19.6%	1.000	4.72±5.57%	0.000	11.24±4.07%	0.000
	HT	37.89±16.59%		0.15±0.02%		23.76±8.70%	
Control group		1.04±0.26%		0.19±0.05%		0.65±0.30%	

*: comparison of T cells subsets in the control group with the mean of T cells subsets in the GD and HT groups.

without a significant difference in comparison to the control group. The plasma cells in GD penetrated thyrocytes but did not damage them. The number of plasma cells was found increased in HT. The CD79 alpha+ B cells constituted almost half of the cells in the mononuclear lymphatic infiltrates. In HT, foci of destruction of thyroid follicles and thyrocytes were visible at the sites of accumulation of plasma cells (Table 3).

Differences were observed under an electron microscope as well. In GD, the lymphocytes were in close contact with thyrocytes but the thyrocytes were not damaged (Fig. 1). Cells with the phenotype of T cells (big, dark nucleus, scanty cytoplasm with ribosomes, and rough endoplasmic reticulum) were present among the thyrocytes in the interstitium and in the wall of the thyroid follicles. Plasma cells with a typical rough endoplasmic reticulum were visible under the basal membrane of the thyroid follicles. It was possible to observe secretion of an electron dense substance (probably antibodies) and its accumulation in the basal membrane of the thyroid follicles (Fig. 2). The surrounding thyrocytes were active with a big nuclei, a well-developed apical pole, numerous secretory vesicles and microvilli on the surface.

In HT, thyrocytes that were in contact with lymphocytes, especially those with large granular cells, exhibited signs of apoptosis. The nucleus was condensed and dominated by heterochromatin. The cisterns of the endoplasmic reticulum were enlarged and the mitochondria were swollen. In some areas, remnants of apoptotic thyrocytes were visible. The basal membrane of the thyroid follicles was thicker because of deposits of collagen fibres. The contact between the capillary vessel lumen and thyrocytes was hampered (Fig. 3).

Discussion

In autoimmune disorders, the ectopic lymphatic tissue organized in lymph follicles is located in the area of non-lymphatic organs, which do not contain a physiologically organized, expanded lymphatic tissue.[17-19] A more intensive proliferation of B and T cells was observed in children with HT than in those with GD. Some authors have suggested that this is probably related to the immunosuppressive action of methimazole used in the treatment of GD.[9,17,19] However, the main action of methimazole in the thyroid consists in decreased activity and increased apoptosis of thyrocytes.[14,15] L-thyroxine used in the treatment of HT exhibited no immunosuppressive activity.[2,20] In our study, the influence of the treatment was difficult

Table 3. Subset sof B-cells in Graves' disease and in Hashimoto's thyroiditis

		CD79 alfa + B cells	P^*
Mean	GD	22.89±8.61%	0.000
	HT	31.65±9.11%	
Parenchyma	GD	6.41±2.35%	0.002
	HT	17.23±8.70%	
Lymphatic infiltrations	GD	39.33±14.00%	1.000
	HT	46.67±17.80%	
Control group		4.11±1.94%	

*: comparison of B cells subsets in the control group with the mean of B cells subsets in the Graves' disease (GD) and Hashimoto's thyroiditis (HT) groups.

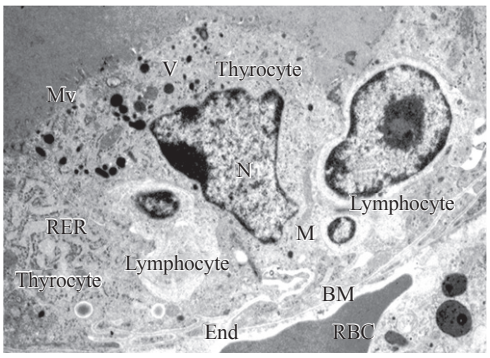

Fig. 1. T-cells between thyrocytes in Graves' disease. The thyrocyte is columnar, active, and does not exhibit signs of damage. N: nucleus; M: mitochondria; Mv: microvilli; RER: rough endoplasmic reticulum; BM: basal membrane; RBC: red blood cell (Original magnification×7000).

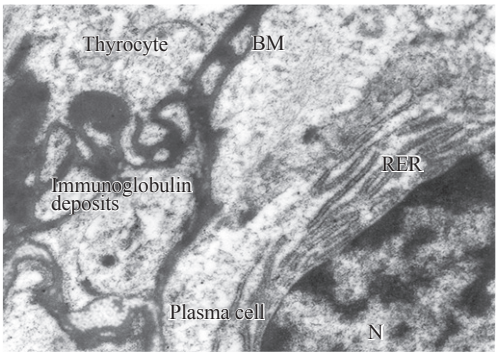

Fig. 2. Deposits of immunoglobulin in the basal membrane in Graves' disease. N: nucleus; BM: basal membrane; RER: rough endoplasmic reticulum (Original magnification×25 000).

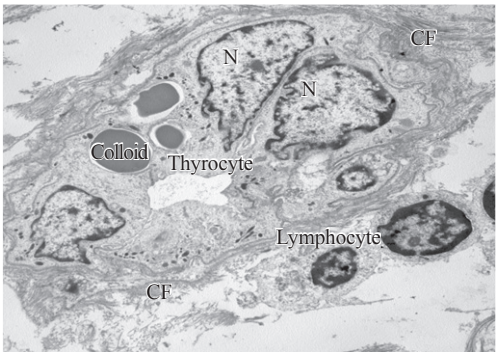

Fig. 3. T cells and plasma cells between thyrocytes in Hashimoto's thyroiditis. Destruction of thyrocytes and signs of apoptosis. Deposits of collagen fibres in the basal membrane. N: nucleus; CF: collagen fibres (Original magnification×7000).

to evaluate, as it was not possible to investigate pre-treatment specimens. After comparison to the previously cited papers, it seems that the changes associated with very intensive immune reactions are predominant in the thyroid.

CD4+ T helper cells were often found in the parenchyma of the thyroid of children with GD. Other researchers, who analyzed lymphocyte subsets in peripheral blood in children with GD, found that the percentage of CD4+ T cells was increased more significantly in patients in the hyperthyroid phase before treatment than in healthy children.[21] CD4+ T cells were present in the lymphatic infiltrations and interstitium. They probably stimulated the humoral response in GD.

Different situation has been described in patients with HT. We observed a decrease in the number of CD4+ T cells in patients with HT as compared those with GD. The number of CD4+ T cells in the interstitium was very low. However it was lower than in the control group. Our findings are consistent with those of previous investigations on animals. Besides, removal of T regulatory CD4+CD25 subsets in healthy rats could lead to spontaneous development of autoimmune diseases: thyroiditis, gastritis, or diabetes.[22] Depletion of CD4+CD25+ T cells has been suggested by Bossowski as the main cause of AITD.[9]

CD8+ T cells were observed in the thyroid follicles in thyrocytes and in lymphatic T cells adjacent to normal active thyrocytes; whereas in HT the contact of CD8+ T cells caused apoptosis of thyrocytes. In patients with HT, hypothyroidism is related to apoptosis of thyrocytes. In our study, electron microscopy revealed remnants of thyrocytes in the lumen of the thyroid follicles. It was discovered that TPO-specific lymphocytes led to thyrocyte destruction because of the cytotoxic mechanism with participation of CD4+ T cells and CD8+ T cells or because of programmed apoptosis with participation of Fas and TNF alpha.[23] Electron microscopy also showed lymphocytes with T cell phenotype and similar localization as that of CD8+ T cells, as well as polarization of endolysosomes at the site of cell contact. Similar observations were made in experimental conditions by Gardella et al.[24] Negrini et al[25] reported a possibility of presence of GITR antigens on the surface CD8+ T cells, which render them characteristics of regulatory cells [Treg]. Lymphocytes CD8+ in GD probably have a regulatory characteristic, as they are located in thyrocytes and do not cause apoptosis.

Extensive immunophenotypic and functional data suggested that CD79a is a B cell-specific antigen.[26-28] Our study and others[29,30] indicated that B-cells were the predominant plasma cells. The number of plasma cells in the thyroid in GD was inversely proportional to the time of treatment, which proved the immunomodulant activity of thyrostatic drugs.[31] Undoubtedly, the high levels of TPO and TG antibodies are the result of the activity of plasma cells in HT. It is probable that autoantibodies themselves do not damage thyrocytes. In the destruction reaction, an important role is played by T cytotoxic cells, killer cells, and natural killer cells in the antibody-dependent cellular cytotoxicity (ADCC) process.[15,16,32] Transfer of HT in experimental circumstances and bone marrow transplantation in humans has been reported.[12-14] Plasma cells in GD are the source of TRAb and other autoantibodies. Transfer of GD through bone marrow transplantation has also been described.[16]

The progressive damage of the thyroid in AITD has not been well elucidated. It seems that the increase in CD4+ T cells and regulatory activity of CD8+ T cells in GD leads to production of antibodies against the TSH receptor and other autoantibodies by plasma cells. Hyperactivity of thyrocytes leads to expression of antigens on the surface of thyrocytes; the cells of the thyroid epithelium play a role of antigen presenting cells.

In HT, thyrocyte damage in the process of ADCC and apoptosis leads to intensification of autoimmune reaction because of release of intracellular antigens, which normally have no contact with lymphocytes. The primary cause is probably the decrease of CD4+ T cells in the thyroid interstitium and activation of CD8+ T cells as cytotoxic cells. The autoimmune reaction in HT leads to activation of fibroblasts and production of collagen fibres, which hinder oxygen and metabolite transport between the lumen of capillary blood vessels and thyrocytes.

In conclusion, the autoimmune reaction in GD involves activation of CD4+ T cells and transformation of B-cells to TRAb-producing plasma cells. The number of CD8+ T cells is increased, but it is not related to the destructive process in the thyroid. The autoimmune reaction in HT consists of the ADCC process in circumstances of a decreased number of CD4+ T cells in lymphatic infiltrations, especially in the parenchyma, compared to GD. CD8+ T cells are involved in the destructive process in the thyroid. The differences between GD and HT suggest that the changes in T-cell subsets may affect the course of these reactions.

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Ethical approval: The investigation was approved by the Ethical Committee at the Medical University in Lublin. All parents and patients signed an informed consent before these investigations.

Competing interest: No conflicts of interest.
Contributors: Ben-Skowronek I proposed the study, collected and analysed the data, and wrote the first draft. All authors contributed to the intellectual content. Szewczyk L and Koroboweicz E are the guarantors.

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