Analysis of Circulating T γ/δ Lymphocytes and CD16/56 Cell Populations in Children and Adolescents with Graves' Disease

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ABSTRACT

The aim of the present study was to determine the proportion of γ/δ T-lymphocytes and CD16/CD56 (CD3 $-$ and CD3 $+$) cells in the peripheral blood of children and adolescents with Graves' disease (GD; $n = 27$; mean age, 15.5 ± 5.1 y) and nontoxic nodular goiter (NTNG; $n = 25$; mean age, 15.2 ± 5.7 y), in comparison with sex- and age-matched healthy control subjects ($n = 25$; mean age, 15.9 ± 2.4 y). In addition, in patients with GD, we investigated the effect of methimazole therapy on the proportion of these cells. We also looked for associations among the parameters investigated. The percentages of γ/δ TCR+CD3 $+$ lymphocytes and CD3 $+$, CD16/56+CD3 $+$, and natural killer (NK) cells were analyzed by the three-color flow cytometry using a Coulter EPICS XL cytometer. In patients with untreated GD, we observed a significant decrease in γ/δ T (CD3 $+$) ($p < 0.002$), CD16/56(CD3 $+$) ($p < 0.001$), and NK ($p < 0.001$) cells in comparison with the healthy control subjects. After 2-6 mo of methimazole therapy, the percentages of γ/δ TCR+CD3 $+$ and CD16/56(CD3 $+$) cells in peripheral blood of hyperthyroid patients returned to the normal values, whereas the percentages of NK cells normalized after 18-24 mo of therapy. These abnormalities were absent in children and adolescents with NTNG. Furthermore, there was no difference in the percentage of CD3 $+$ lymphocytes in all of the groups. In the patients with untreated GD, we found a negative correlation between free

thyroxine concentration in blood serum and the percentages of CD16/56 (CD3 $-$) and γ/δ T cells ($r = -0.5$, $p < 0.035$; $r = -0.4$, $p < 0.02$). No such correlation was detected in patients with NTNG. We conclude that the abnormal distribution of CD16/CD56 (CD3 $-$ and CD3 $+$) cells and γ/δ T lymphocytes in the peripheral blood in children and adolescents with untreated GD suggests their role in the development of autoimmunity. (*Pediatr Res* 54: 425-429, 2003)

Abbreviations

AITD, autoimmune thyroid disease
ft3, free triiodothyronine
ft4, free thyroxine
GD, Graves' disease
hTSH, human thyroid-stimulating hormone
NK cells, natural killer cells
NTNG, nontoxic nodular goiter
PE, phycoerythrin
PerCP, peridinin chlorophyll protein
TG-Abs, antithyroglobulin antibodies
TPO-Abs, antithyroid peroxidase antibodies
TRAb, antibodies against receptor for thyroid stimulating hormone

Graves' disease (GD) is an autoimmune thyroid disease (AITD) characterized by the production of thyroid autoantibodies against the TSH receptor, symporter Na $^{+}/^{-}$, thyroglobulin or thyroperoxidase antigen, and lymphocytic infiltration of the thyroid gland (1, 2). The immune cells [lymphocytes γ/δ T-cell receptor (TCR), CD16/56(CD3 $+$), and natural killer (NK) cells] may play a pivotal role in the development of AITD (3-5).

T cells that bear the γ/δ TCR (formed by γ and δ chains) in peripheral blood represent a minor population of T cells (2-10%) (6). In mouse, these cells specifically migrate in an orderly way to the skin and to mucosa of the respiratory, gastrointestinal, and genital tracts (7). In humans, this distribution in tissues has been reported in inflammatory and autoimmune sites such as synovium in rheumatoid arthritis and cerebrospinal fluid and inflammatory intestinal tissue in multiple sclerosis (8-10). Human γ/δ T cells have been shown to recognize mycobacteria antigen, heat shock proteins, and low-weight antigens without restriction to major histocompatibility complex class I or II of the presented molecules. These cells reveal cytotoxic and NK's capabilities and produce cytokines such as tumor necrosis factor- α and γ -interferon (6, 11). The

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role of the respective populations of lymphocytes in the pathogenesis of GD in children has not been fully elucidated, and data concerning the percentage of $\gamma\delta$ T cells in the peripheral blood are not unequivocal. Results presented by Sasian *et al.* (4) showed a decrease of $\gamma\delta$ T lymphocytes in patients with untreated hyperthyroidism as a result of the action of excessive thyroid hormones or accumulation of these lymphocytes in the target organ.

A substantial role also can be ascribed to subpopulations of CD16/56+ lymphocytes, the so-called cytotoxic (with CD3+) cells and NK (with CD3-) cells (5). Numerous studies have revealed disturbances in the distribution and function of these subpopulations in adults in several immune diseases, including lupus erythematosus, multiple sclerosis, and thyroid autoimmune diseases (12–14). Deficiency in CD16/56 (CD3+ and CD3-) cells in patients with GD may result in the decrease of cytolytic activity, which is related to hyperthyroxinemia (15).

In the present study, we determined the percentages of $\gamma\delta$ TCR+CD3+ lymphocytes and CD3+, CD16/56+CD3+, NK cells in the peripheral blood in children and adolescents with newly diagnosed GD before and after 2–6 mo and 18–24 mo of methimazole therapy, in levothyroxine-suppressed patients with nontoxic nodular goiter (NTNG), and in healthy control subjects, to elucidate the relationship with circulating antithyroid antibodies and thyroid hormone concentrations and a possible effect of methimazole therapy on the proportion of the above cells.

PATIENTS AND METHODS

We studied 52 patients (10 boys and 42 girls) aged 8–19 y (mean age, 15.3 ± 5.4) with GD ($n = 27$ before and $n = 27$ after methimazole therapy; mean age, 15.5 ± 5.1) and NTNG ($n = 25$; mean age, 15.2 ± 5.7), and a group of 25 sex- and age-matched healthy control subjects with no personal or family history of the thyroid and autoimmune disease (2 boys and 23 girls; mean age, 15.9 ± 2.4). Healthy control subjects were euthyroid, with negative antithyroid antibodies. None of the subjects had experienced acute infection or other illness during the period of 8 wk before sample collection. The diagnosis was based on clinical examination, laboratory tests, and ultrasonography of the thyroid gland. In patients with nodular goiter, fine-needle biopsy was also performed. In all of the cases, the changes were classified as benign (nodular goiter). All of the patients met the following criteria: 1) absence of clinical evidence of disease other than thyroid disease, 2) no history of systemic diseases, 3) no application of immunoactive drugs, and 4) normal liver and kidney function.

Schedule of study. Analysis of lymphocyte markers was performed at the first visit. Then, patients with GD were treated with methimazole, at an initial dose of $1 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ (maximum, $30 \text{ mg} \cdot \text{m}^{-2} \cdot \text{d}^{-1}$) in combination with propranolol ($0.5\text{--}1 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$). Once clinical euthyroidism had been obtained (second evaluation of lymphocytes, between 2 and 6 mo), methimazole doses were reduced by 5–10 mg to reach the maintenance dose of 5–10 mg. Levothyroxine was added if hypothyroidism appeared. Twenty-seven patients with GD achieved control of hyperthyroidism by receiving methim-

azole for 18–24 mo and remained euthyroid (third period of lymphocyte reevaluation). Patients were followed up for 36 mo after the diagnosis. Patients with NTNG received suppressive doses of thyroxine (mean, $75 \pm 25 \mu\text{g/d}$) for the period of 6–18 mo (mean, 13 mo).

Blood samples were collected between 0730 and 0900 h for the measurement of serum free triiodothyronine (fT3), free thyroxine (fT4), human thyroid-stimulating hormone (hTSH), and antithyroid antibodies and (in EDTA tube) for morphologic parameters and CD phenotyping. All parents of patients and control subjects gave informed consent before enrollment. The protocol for the study was approved by the Local Ethical Committee at the BiaŁystok Medical Academy.

Immunologic marker analysis. The peripheral blood samples were analyzed following the previously described techniques (16). Three-color immunophenotyping of lymphocytes was performed by a lysed whole-blood method using combinations of mouse MAb directly conjugated to Simultest (Becton Dickinson Immunocytometry System, San Jose, CA, U.S.A.). All samples were processed using the Q-Prep whole-blood lysis system (Beckman Coulter, Inc., Fullerton, CA, U.S.A.). Briefly, 100- μL samples of whole blood were stained with 10 μL of the MAb (Simultest) directly conjugated to FITC, phycoerythrin (PE), or peridinin chlorophyll protein (PerCP): CD3-PerCP(Leu-4), CD3-PerCP(Leu-4)/CD16/CD56-PE, CD3-PerCP/ $\gamma\delta$ TCR-PE. After incubation at room temperature for 20 min, the samples were then processed with a 35-s cycle on a rapid no-wash whole-blood lysis workstation. A minimum of 10^4 cells were analyzed by Coulter EPICS XL flow cytometer. The percentage of positive cells was determined by setting the lower limit over the nonspecific fluorescence with a suitable control.

Thyroid hormones and autoantibodies. Serum levels of anti-thyroid-stimulating hormone receptor antibodies (TRAbs) were routinely determined by a radio receptor assay (TRAK-Assay; Brahms Diagnostica, Berlin, Germany). The test is based on the binding of the TSH receptor with immunoglobulins present in blood serum and directed against this receptor and with radioiodine-labeled TSH (^{125}I -TSH). TRAb values $<9 \text{ U/L}$ were considered TRAb negative; TRAb concentrations $>14 \text{ U/L}$ were positive. The range between 9 and 14 U/L was defined as borderline (gray zone). The sensitivity of the TRAK-Assay was $<2.4 \text{ U/L}$. Intra- and interassay coefficients of variation were 5.1% and 10.2%, respectively.

Anti-peroxidase antibodies (TPO-Abs) and antithyroglobulin antibodies (TG-Abs) were determined in the sera using immunodiagnostic test Varelisa (Variable Enzyme Linked Immno Sorbent Assay, Pharmacia Upjohn Diagnostics, Freiburg, Germany). These tests use human TPO antigen for anti-TPO assay and human thyroglobulin as antigen for anti-TG determination. Results were read on a photometer (STAT FAX 303 PLUS, ANALCO-GBG) at 450-nm wavelength, with absorption values proportional to the level of anti-TPO or anti-TG antibodies. Normal range for TG-Abs was $<500 \text{ IU/mL}$ and for TPO-Abs was $<50 \text{ IU/mL}$. The levels of fT4, fT3, and hTSH in blood serum were determined on a mini-analyzer (Bio Merieux, Charbonnieres les Bains, France), based on test Varelisa (Pharmacia Upjohn Diagnostics GmbH

& Co. K.G. Freiburg, Germany), combining the immunoenzymatic method with the final fluorescence measurement (ELFA). Normal range of fT4 was 0.71–1.55 ng/dL, for fT3 was 2.6–5.4 ng/L, and for hTSH was 0.32–5.0 μ IU/mL.

Statistical analysis. The results were expressed as the means $\pm$ SD. Comparison of the percentages of $\gamma\delta$ T lymphocytes and CD16/CD56 populations between the groups was carried out using *t* test, confirmed by Mann-Whitney *U* test. *T* value was considered statistically significant at $p < 0.05$. For correlation analysis, Pearson's and Spearman's tests were used. Statistical calculations were based on Statistica 5.0 StatSoft.

RESULTS

The patients with untreated GD had low levels of TSH (mean, 0.19 ± 0.52 μ IU/mL), high thyroid hormone levels (fT3: mean, 13.5 ± 9 ng/L; fT4: mean, 3.11 ± 1.5 ng/dL), and elevated levels of antithyroid antibodies (TRAb: mean, 21.4 ± 10 U/L; TPO-Ab: mean, 1889 ± 1550 IU/mL; and TG-Ab: mean, 1980 ± 1544 IU/mL). Eighteen patients with NTNG were clinically and biochemically euthyroid, with the following mean hormone levels: fT3, 3.24 ± 0.25 ng/L; fT4, 1.17 ± 0.25 ng/dL; and hTSH, 1.43 ± 0.46 μ IU/mL. These patients also had normal concentration of antithyroid antibodies: TPO-Ab, 60 ± 22 IU/mL; TG-Ab, 83 ± 51 IU/mL; and TRAb, 1.46 ± 1.3 U/L.

The analysis of $\gamma\delta$ TCR+ antigen expression on peripheral blood lymphocytes revealed decreased percentages of these positive cells in patients with hyperthyroidism before thyrostatic treatment, compared with healthy control subjects and euthyroid patients with GD. After 2–6 and 18–24 mo of methimazole treatment, the percentages of $\gamma\delta$ T cells were within the normal ranges. These abnormalities were absent in children with NTNG (Fig. 1). In addition, the levels of CD3+ T cells were similar in all of the groups, and no statistically significant differences were found in comparison with healthy control subjects (Table 1).

Patients with GD showed a decrease in the percentage of both CD16/CD56 (CD3– and CD3+) cells, compared with patients with NTNG and healthy control subjects. After 2–6 mo of methimazole treatment, the percentage of CD16/CD56 (CD3+) cells normalized, whereas the percentage of CD16/56

(CD3–) cells was still significantly lower than in healthy children. However, after 18–24 mo of therapy, when patients with GD were in long-term clinical and biochemical remission, the percentages of NK cells returned to normal. There was no significant difference between the proportions of these CD16/56 cell populations in patients with NTNG and healthy control subjects (Table 1).

We studied the correlation between the concentration of thyroxine and percentages of $\gamma\delta$ T lymphocytes and CD16/56 (CD3–), CD16/56 (CD3+) cells. In the patients with untreated GD, we found a negative correlation between fT4 concentration in blood serum and the percentages of $\gamma\delta$ T and CD16/56 (CD3–) cells ($r = -0.5$, $p < 0.035$; $r = -0.4$, $p < 0.02$). We observed no relationship between serum levels of antithyroid antibodies and percentages of $\gamma\delta$ T lymphocytes and CD16/56 (CD3–), CD16/56 (CD3+) cells. No correlation was detected in patients with NTNG.

DISCUSSION

The course of AITD is associated with the inflow of lymphocytes T and B to the thyroid gland and with the expression of anti-TSH receptor, antithyroglobulin, antithyroid peroxidase, and anti-sodium-iodine symporter antibodies (2, 3, 17). This autoimmune process is probably initiated by the activation of autoreactive T cells by a major histocompatibility complex II/self-antigen complex and a co-stimulatory signal (B7/CD28-CTLA-4) that enables full activation of T cells by the expression of surface antigens and cell adhesion molecules (lymphocyte function-associated antigen-1, very late antigen-4, and intercellular adhesion molecule-1), which allow the homing of these immune cells into the target organs (18, 19). In our study, we performed analysis of the circulating populations of immune cells because the clinical, biochemical, and immunologic disturbances in GD are not solely limited to the thyroid gland.

We found a significant decrease in the level of $\gamma\delta$ T cells in the peripheral blood of children and adolescents with newly diagnosed GD, but normal levels of these lymphocytes were registered in subjects after 2–6 and 18–24 mo of methimazole therapy. Our observation is in agreement with the recently published study of Sasian *et al.* (4), who found lower levels of $\gamma\delta$ T cells in patients with GD treated for thyrotoxicosis in comparison with subjects who were in long-term remission. In addition, the authors underlined that changes in the number of $\gamma\delta$ T cells could not be related to the thyroid hormone concentrations and did not normalize after methimazole or radical therapy. However, in our study, we found a significant correlation between the level of thyroid hormones and the percentage of $\gamma\delta$ T cells in children with untreated hyperthyroidism. This relation could indicate that the abnormal thyroid status may lead to systemic immune alterations. However, McIntosh *et al.* (20) did not observe such alterations in patients with hyperthyroidism, and the level of $\gamma\delta$ T cells in the peripheral blood was normal. It is interesting that despite abnormal distribution of $\gamma\delta$ T cells in the peripheral blood, Santamaria *et al.* (21) found a higher proportion of $\gamma\delta$ T cells among the lymphocytes infiltrating the thyroid glands. This observation, together with the findings of Roura-Mir *et al.* (22), could

Figure 1. The percentage of $\gamma\delta$ + T lymphocytes on peripheral blood in patients and in the control group. Patients with GD: group A, before treatment (untreated hyperthyroidism); group B, after 2–6 mo of methimazole treatment; group C, after 18–24 mo of therapy; group D, patients with NTNG; group K, healthy children. Values are means $\pm$ SD. *Group A vs control subjects.

Table 1. Percentages of peripheral blood lymphocyte subsets in healthy control subjects and in patients with GD (before and after 2–6 and 18–24 mo of methimazole therapy) and in NTNG

	GD			NTNG (n = 25)	Healthy controls (n = 25)
	Untreated (n = 27)	After 2–6 mo (n = 27)	After 18–24 mo (n = 27)		
CD16/56(CD3–) (%)	8.26 ± 3.9* <0.001	8.84 ± 5.2* <0.01	13.9 ± 5.2	10.5 ± 5.7	13.3 ± 4.37
CD16/56(CD3+) (%)	2.1 ± 0.8* <0.001	3.16 ± 3.74	3.55 ± 2.9	3.25 ± 3.84	4.4 ± 1
CD3+ (%)	70.2 ± 5.1	75 ± 5.8	69.7 ± 5.9	71 ± 9.4	71 ± 5.3

Corrected values in untreated GD.

Values are means ± SD and in percentages for lymphocyte subsets.

* Statistically significant differences between patients with GD and healthy control subjects.

Marked significant differences using “***”.

suggest abnormal distribution of $\gamma\delta$ T cells in different compartments of the immune system. In addition, the accumulation of $\gamma\delta$ T lymphocytes has been revealed in the affected organs in other autoimmune diseases, such as multiple sclerosis and rheumatoid arthritis (8, 9). It could be suggested that $\gamma\delta$ T cells play an important role in the etiopathogenesis of the autoimmune process. We believe that the decrease in $\gamma\delta$ T cells in the peripheral blood could probably be associated with increased migration of these lymphocytes to the thyroid gland or result from primary defect of the immune system, which leads to autoimmunity.

In the present investigation, we also noted a significant decrease in CD16/56 (CD3–) and CD16/56 (CD3+) cells in the peripheral blood in children and adolescents with untreated GD, whereas in the euthyroid state, the levels of these cells were normal. Our findings are in agreement with the studies of Rojano *et al.* (5), although they observed that the percentages of CD16/56 cell populations after thyrostatic therapy were still lower in comparison with healthy control subjects. In addition, the authors underlined the role of these subpopulations of cells as a prognostic marker of GD. However, Corrales *et al.* (23) did not reveal any statistically significant changes in the percentage of CD16/56 (CD3–) cells in the peripheral blood of patients with GD in comparison with control subjects and in hyperthyroid patients who were in long-term remission and those with relapse. It is interesting that despite abnormal distribution of NK cells in the peripheral blood, Aust *et al.* (24) found a lower proportion of CD16/56 (CD3–) cells among the lymphocytes infiltrating the thyroid gland. These data suggest that the reduction in the number of NK cells could be a consequence of the predominant role of Th2 cell clones (humoral immune reactivity) in the pathogenesis of GD, stimulating B lymphocytes to the production of TSH receptor antibodies. Moreover, Tezuka *et al.* (25) speculated that the loss of NK cell activity in thyroid gland contributes to the perpetuation of GD by allowing the immune response to proceed without feedback suppression.

Our present study has also shown a significant negative correlation between thyroid hormone level and the percentage of CD16/56 (CD3–) cells in the peripheral blood of patients with GD. This observation is in agreement with the published study of Corrales *et al.* and others (15, 26, 27), who underlined that the decrease in cytolytic activity in patients with hyper-

thyroidism could be a consequence of a metabolic effect of thyroid hormones on the number and function of CD16/56 cell populations. Abnormal distribution of NK cell subsets was also observed at the diagnosis of different autoimmune diseases such as multiple sclerosis and lupus erythematosus and was associated with the onset of clinical symptoms of the disease (12, 13).

In the present study, methimazole therapy led to clinical-biochemical remission after 8–12 wk of treatment. The levels of antithyroid antibodies and the percentages of CD16/56 (CD3+) and $\gamma\delta$ T cells were normalized after 2–6 mo of treatment, whereas the percentages of CD16/56 (CD3–) cells returned to normal after 18–24 mo of methimazole therapy. The above data indicate strong autoimmune stimulation of the thyroid gland (a long time is required to normalize the percentage of NK cells) in children and adolescents with GD. Moreover, normalization of disturbances in the percentage of CD16/56 (CD3–), CD16/56 (CD3+) cells, and $\gamma\delta$ T lymphocytes and reduction in serum levels of antithyroid antibodies seem to prove the immunomodulatory effect of methimazole.

CONCLUSION

We conclude that the abnormal distribution of CD16/CD56 (CD3– and CD3+) cells and $\gamma\delta$ T lymphocytes in the peripheral blood in children and adolescents with untreated GD suggests their role in the development of autoimmunity.

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Erratum

In the article “Kinematic quality of Reaching Movements in Preterm Infants” by B Fallang et al (*Pediatr Res* 53: 836–842, 2003) there is an error in Figure 1, “Lowrisk preterm section”. The corrected figure appears below. The authors regret this error.