ALTERED EXPRESSION OF T LYMPHOCYTE SURFACE MARKERS IN CHILDREN WITH CHRONIC AUTOIMMUNE THYROIDITIS

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CTLA-4 and CD28+ are regulators of T cell activation. The CTLA-4 gene is associated with variety of autoimmune diseases. The aim of the study was to evaluate changes in basic T cell subpopulations, and the expression of CD152+ and CD28+ before and after T cell stimulation in children with autoimmune thyroiditis (AT), as compared with control subjects. Blood samples were obtained from 35 AT children and 25 healthy children. CD markers were evaluated by flow cytometry at baseline, after the culture with phytohemagglutinin and without stimulation. At baseline, CD152+ expression was lower in patients than in controls (P<10^-6). After stimulation, there were an increase in CD152+ T cells and decreases in CD28+ and CD4+ cells in controls (P<0.01). In AT children, CD152+ T cells remained stable. CD4+CD152+ T cells correlated inversely with antithyroglobulin antibodies. We conclude that alterations in lymphocyte markers are associated with AT. Stimulation leads to differing changes in T-lymphocyte subsets in both examined children populations.

Key words: autoimmune thyroiditis, children, CTLA-4, T cells

INTRODUCTION

The CTLA-4 (CD152+) and CD28+ are among the main regulators of T cell activation, and are the two homologous members of the immunoglobulin superfamily. They bind to the same ligands, CD86+ (B7-2) and CD80+ (B7-1), expressed on antigen presenting cells (APCs), but the function of CD28+ and CTLA-4 is different. CD28+ is a positive co-stimulator and CTLA-4 is a negative regulator of T cell activation. CTLA-4 has a much higher binding affinity for CD86+ and CD80+ than that for CD28+ (1). CD28+ is constitutively expressed on a naive
CD4+ T cells and is slightly upregulated after T cell activation. The CTLA-4 protein is primarily localized in intracellular vesicles and cycles between intracellular stores and the surface of T cell, which occurs through the interaction of the clathrin-associated protein complex AP-2. Surface expression of CTLA-4 increases after T cell activation and peaks after 48-72 h. Only does a fraction of activated T cells express it on the cell surface (2). Naive T cells express the CTLA-4 only after activation, but naturally occurring regulatory T cells (T regs) constitutively express this molecule (3). The basic function of CTLA-4 and CD28+ is the control of T cell activation and apoptosis (4), but they also contribute to the maintenance of peripheral tolerance (5, 6) and the control of T cell differentiation and maturation (7, 8). The normal balance between CD28+ and CD152+ ensures the normal homeostasis and function of T cells (9). The importance of this co-stimulatory pathway was studied in several experiments in genetically engineered mice. In CD28+ -deficient mice T cells differentiated normally, but were functionally impaired (10). CTLA-4-deficient mice suffer from lymphoproliferative disease with early lethality (11, 12). Additionally, the CTLA-4 gene was found to be associated with a variety of autoimmune diseases, like type I diabetes mellitus (13), autoimmune thyroid diseases (14), and with bronchial asthma (15).

The aim of the present study was to evaluate changes in the basic T cell subpopulations: CD4+ and CD8+, and the expression of CD152+ and CD28+ on T cells before and after in vitro stimulation of T cells from children with the chronic autoimmune thyroiditis in comparison with healthy controls.

MATERIAL AND METHODS

The study was approved by the Bioethics Committee of Warsaw Medical University, Warsaw, Poland. Parents of the patients signed informed consent for the participation in the study.

The blood samples were obtained from 35 children with the chronic autoimmune thyroiditis (AT) or Hashimoto's thyroiditis and from 25 healthy children, age and sex matched, free of allergic, immune and hematological disorders, and with a normal thyroid gland function. The diagnosis of AT was based on the presence of anti-thyroid antibodies and the characteristic picture of the thyroid gland on ultrasonographic examination. In the group of patients with AT, the mean age was 14.8 ±2.35, and female to male proportion was 31/4. In the control group, the mean age was 14.6 ±2.25, and female to male proportion was 21/4.

The anti-thyroid antibodies were measured by the Microparticle Enzyme Immunoassays AxSYM Anti-Tg and AxSYM Anti-TPO (Abbott Laboratories, Abbott Park, IL).

Cell preparation

Heparinized blood samples from AT children and healthy controls were diluted three times with saline and centrifuged for 30 min by 400 x g on Histopaque 1077-1 density gradient from SIGMA Diagnostics (Saint Louis, MO). The isolated peripheral blood mononuclear cells (PBMC) were washed three times with saline and suspended in phosphate-buffered saline (PBS). The isolated PBMC were incubated with monoclonal antibodies for 30 min at 25°C in the darkness. Analysis was performed with the use of monoclonal antibodies combination: CD4-FITC/ CD28 -PC5/ CD152-PE.
and CD8-FITC/CD28-PC5/CD152-PE obtained from Immunotech Beckman Coulter Company (France). After incubation, samples were fixed and lysed by the reagent set Uti-Lyse (Dako Cytomation, USA). The T cell phenotype was evaluated using the flow cytometer Beckman Coulter EPICS XL 4C (EPICS XL/XL-MCL, version 2.0).

Cell culture

Isolated PBMC were suspended in the medium in the concentration 2 x 10⁶ cells per 1 ml of the medium. Culture medium was prepared with the use of reagents of Sigma Chemical Co, USA. PBMC suspension was cultured with or without phytohemagglutinin (PHA). The concentration of PHA was 20 µg per 1 ml. The culture was run for 48 h at 37°C in humid chamber with controlled 5% inflow of CO₂. PBMC were then washed with PBS and centrifuged for 10 min at 1800 cycles/min, resuspended in PBS, and marked with proper monoclonal antibodies. The results were estimated by a flow cytometer.

Data analysis

There were three time points of T cell phenotype evaluation: at the start of experiment, after 48-h culture with phytohemagglutinin (PHA), and after 48-h culture under the same circumstances without the PHA stimulation. The results were statistically analyzed by a Mann-Whitney U-test, using STATISTICA XL7.0 software package.

RESULTS

At baseline, a significant difference between T cells phenotypes of AT children and healthy controls was detected only in the expression of CD152+. In AT children, the surface expression of CD152+ was significantly lower than in healthy subjects; the mean percentages were 2.6 ± 1.8% vs. 4.5 ± 1.5% (P=10⁻⁸). The alterations were evident in both basic T cell subpopulations: CD4+ and CD8+ T cells. The mean percentages of CD4+CD152+ T cells were 1.0 ± 0.8% in AT children and 2.5 ± 1.6% in controls (P=0.0001). The mean percentages of CD8+CD152+ T cells were 1.2 ± 1.6% in AT vs. 2.5 ± 2.0% in healthy children (P<0.0004) (Fig. 1).

After a 48-h culture of the purified T cell samples stimulated with PHA, different alterations in T cell phenotypes were noted in AT and control children. We compared the percentages of T cell phenotypes in cultures after stimulation with those present without stimulation, performed simultaneously as a control. In healthy children, stimulation significantly increased the number of CD152+ T cells (6.1 ± 0.9% vs. 4.3 ± 1.6%; P=0.01). That increment was due to the elevation of a percentage of CD8+CD152+ T cells: 4.0 ± 1.5% vs. 2.2 ± 1.5%, P=0.04). Furthermore, PHA stimulation in healthy children decreased CD28+ T cells (12.1 ± 4.5% vs. 31.7 ± 3.8%; P=10⁻⁸) and CD4+ T cells (15.6 ± 5.1% vs. 25.2 ± 3.9%; P=10⁻⁸). The percentage of CD8+ T cells also decreased in comparison with the control T cell culture without stimulation (14.2 ± 4.4% vs. 19.6 ± 8.8%; P=0.02). The proportion CD4+/CD8+ after stimulation was 1:1.
In AT children, the number of CD152+ T cells after the 48-h culture with PHA stimulation was on the same level as that in T cell culture without stimulation (4.13 ± 2.2% vs. 4.3 ± 2.7%). CD28+ T cells decreased (13.0 ± 7.1% vs. 39.7 ± 10.3%; P=10^-7). CD4+ T cells also decreased (21.8 ± 11.4% vs. 29.2 ± 11.1%; P=0.05). The proportion CD4+/CD8+ T cells after stimulation in AT children was 1.8:1.0.

The comparison of the CD profiles in AT and healthy children revealed that after the 48-h PHA stimulation the percentage of CD152+ T cell was lower in the
former (4.1 ± 2.2% vs. 6.1 ± 0.9%, respectively; P=0.01). CD28+ T cells decreased in both groups and did not differ between them significantly before and after stimulation (13.0 ± 7.1% in AT children vs. 12.1 ± 4.5% in controls) (Fig. 2). In AT children, after stimulation, the CD4+ T cell subset was significantly greater than that in healthy controls: 1.8 ± 11.4% and 15.6 ± 5.1%, respectively; (P=0.01) and the CD4+/CD8+ ratio was higher in AT children than that in controls (1.8:1.0 vs. 1:1), but this difference was not statistically significant (P>0.05).

Finally, we found that the percentage of CD4+CD152+ T cells correlated negatively with the antithyroglobulin antibodies level (r=-0.34; P=0.04); an association that could be of potential clinical importance considering the difference between the T cell subsets in AT and healthy children.

**DISCUSSION**

The knowledge on CTLA-4 expression is based mainly on animal models. Despite a high degree of the sequence conservation in the mouse and human CTLA-4 genes and proteins (16), the control of CTLA-4 expression in both species seems to differ. In the mouse, the CTLA-4 mRNA is present only in activated T cells (17), but in man, non-activated T cells express the CTLA-4 mRNA and CTLA-4 protein (18). The difference is also evident following the T cell stimulation. According to the studies based on T cell activation in vitro, the maximal expression of CTLA-4 protein is reached after 2-3 days (19-21). Some reports suggest that the proportion of intracellular and surface CTLA-4 remains unchanged, which may reflect similar changes in its expression due to activation in both loci (19, 22).
The results of our study showed that in children with chronic autoimmune disease the expression of CTLA-4 on T cells is impaired at baseline, but the percentages of basic T cell subsets are comparable with those in the control group. The difference between the two groups increased after T cell stimulation. In AT children, CTLA-4 expression remained on the same low level, whereas it significantly increased in healthy individuals.

Wang et al (18) analyzed the surface and intracellular expression of CTLA-4 in 20 healthy individuals. On the average, CTLA-4 was expressed on the surface of 1.95% of CD4+ and 2.40% of CD8+ human resting T cells (18). In our present material, the percentages of CTLA-4 were similar. After the stimulation with IL-2 in the human PBMC culture, the intracellular and surface expression of CTLA-4 significantly increased with a peak level after 2 days. CD28+ expression was slightly decreased in healthy individuals after the activation (18). Our results also support this observation. The expression of CD28+ decreased after a 48-h PHA stimulation in both examined groups.

Another significant difference considered changes in the CD4+/CD8+ ratio, with the predominance of CD4+ T cells in AT children. It may suggest that the impairment of the CTLA-4 expression caused this shift in the basic T cell subsets. The results correspond with those in a study by Yu et al (9). These authors showed that interactions between B7 and CD28+/CTLA-4 co-stimulatory receptors have a substantial functional role in the regulation of CD4+ and CD8+ T cell homeostasis. They analyzed the expression of CD4+ and CD8+ in the thymic and peripheral T cells in transgenic mice with overexpression of B7 (B7 Tg mice) and in B7 deficient mice (B7 def. mice). B7 Tg mice had a higher percentage of CD8+ cells and a lower one of CD4+ cells than those present in the transgene-negative littermates. The CD4+/CD8+ ratio was reduced in B7 Tg mice. These results indicate that an overexpression of B7 can lead to a shift in the CD4+/CD8+ balance in the thymus and, more notably, in peripheral T cell compartments, favoring an increased proportion of CD8+. These authors also evaluated the expression of CTLA-4. The surface CTLA-4 expression in their experiment was induced by an in vitro activation. The B7 Tg mice had increased cytoplasmic expression of CTLA-4 in CD4+ and CD8+ T cells. The authors conclude that the surface CTLA-4 may contribute to preferential down-regulation of CD4+ and the observed skewing toward CD8+ cells in B7 Tg mice. In contrast, in B7-def. mice, the CD4+/CD8+ ratio was notably increased in comparison with controls. The percentage of CD4+ T cells and the CD28+ expression was higher in B7 def. mice (9). These changes suggest a key role for B7/CD28+/CTLA-4 pathway in regulating the development and homeostasis of CD4+ and CD8+ T cells.

In recent years, a great progress has been made in describing and identifying several types of regulatory T cells (T regs) (23, 24). They play an important role in the control of the peripheral tolerance. The best known naturally occurring T regs belong to the CD4+ subset, and express CD25+ on the cell surface. They comprise approximately 10% of peripheral CD4+ T cells in humans. The
CD4+CD25+ T regs constitutively express also CTLA-4, glucocorticoid-induced tumor necrosis factor receptor family related gene (GITR) and FOXP3 (23). The impaired expression of CTLA-4 could have serious consequences in the peripheral tolerance maintenance and plays an important role in autoimmune diseases in human (24, 25).

The experiments of Tivol et al (11) and Waterhouse et al (12) showed that CTLA-4 plays a key role in T cell activation and proliferation. CTLA-4 deficient mice express a phenotype of profound peripheral lymphoproliferation with the predominant CD4+ T cell expansion and continuous activation of T cells. The extensive expansion of CD4+ T cells that occurs in CTLA-4 deficient mice suggests that CTLA-4 preferentially inhibits activation and expansion of CD4+ and that the CD28+ favors the CD8+ expansion. In our study, the CTLA-4 deficiency was confirmed at both baseline and after T cell stimulation in patients with chronic autoimmune thyroiditis. The CTLA-4 expression correlated inversely with the level of antithyroglobulin antibodies, which are one of the hallmarks of autoimmune thyroiditis. Our results suggest that CTLA-4 disorders could be responsible for the development of autoimmune thyroiditis in human.

In conclusion, alterations in lymphocyte surface markers are associated with autoimmune thyroiditis in children. Lymphocyte activation in both examined populations leads to differing changes in the proportion of T cell subsets. Defective CTLA-4 expression on activated T cells may promote the activation of co-stimulatory T cell signaling pathways leading to autoimmune diseases, including autoimmune thyroiditis. This effect may be primary or result from abnormal immune regulation.

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