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Th1/Th2 Cytokines in Patients with Graves' Disease with or without Ophthalmopathy

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ABSTRACT

About 25-50% of Graves' disease (GD) patients develop thyroid eye diseases, which is associated with inflammatory process and abnormalities in the levels of several cytokines in orbital tissues in GD. The aim of this study was to determine the Th1 and Th2 serum cytokines in patients with GD with or without ophthalmopathy.

Serum levels of cytokines and autoantibodies including Interferon-gamma (IFN-γ), Interleukin-2 (IL-2), Interleukin-4 (IL-4), Interleukin-10 (IL-10), TSH receptor autoantibody (TRAb), thyroid peroxidase antibody (TPOAb) and thyroglobulin antibody(TgAb) were measured by enzyme linked immunosorbent assay(ELISA) in 34 patients with GD and in 33 normal controls. Patients were also divided in two subgroups: 18 cases with ophthalmopathy and 16 cases without ophthalmopathy. Cytokine and antibody responses were analyzed in both groups.

Compared with control subjects, patients with GD showed elevated levels of IL-2 and IL-10. IFN-γ levels were lower in patients in comparison to the controls. No significant differences were found between patients and controls regarding the IL-4. There was no statistically significant difference in cytokine levels between those with or without ophthalmopathy.

Quantitative-cytokine analysis demonstrated that a combination of Th1 and Th2 cytokines may contribute to the pathogenesis of GD. These results also indicate that IL-10, but not IL-4, is related to the moderate and severe forms of thyroid associated ophthalmophathy.

Keywords: Cytokines; Graves' disease; Ophthalmopathy; T-Lymphocytes

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INTRODUCTION

Graves' disease is an organ-specific autoimmune disorder, characterized by diffuse goiter, thyrotoxicosis and infiltrate orbitopathy.[1,2] Although the etiology of GD is obscure and the series of events leading to GD are not fully understood, it is well established that genetic, environmental, and immunological factors contribute to the pathogenesis of disease.[3-5] Several studies have indicated that T cell activation and abnormal cytokine production may contribute to clinical manifestations of GD.[4,6] Cytokines are potent mediator and communication molecules, influencing a wide range of biologic functions, such as immune responses.[7]

Whereas the role of cytokines in the pathogenesis of a number of human disorders is not yet identified, assays for cytokines have become a popular procedure in research and medical laboratory to initially find out whether there is alteration in the production of cytokines in these disorders. To date, a number of researchers are proposed that altered balance between T helper type 1 and 2 (Th1 and Th2) cells, may play an important role in the pathogenesis of GD.[8,9] However, there is controversy in the literature about GD being a Th1 (cell-mediated), or Th2 (humoral) autoimmune phenomenon, or both. In other words, the role of cytokines in systemic circulation and their contributions in GD pathogenesis remains ambiguous.

There are some limitations in the study of kinetic changes of cytokines in the eyes during the development of graves' ophthalmopathy (GO).

Table 1. The NOSPECS* classification of ocular changes in thyroid-associated ophthalmopathy

Class	Definition
0	No signs or symptoms
1	Only signs, no symptoms
2	Soft tissue involvement
4	Extraocular muscle involvement
4	Extraocular muscle involvement
5	Corneal involvement
6	Sight loss

*NOSPECS is an acronym representing the first letter of each definition and are indicated by bold characters. Classes 1 to 6 are then further subdivided into four subclasses, absent, mild, moderate, or marked.

The restrictions such as the difficulties in obtaining tissue specimens from patients with thyroid-associated ophthalmopathy, the technical problems associated with small specimen size and the patchy distribution of the lesion are in part responsible for conflicting study outcomes. As a result, we decided to quantify a panel of cytokines in sera of these patients.

The aim of this study was (i) to determine whether there is a difference in cytokine levels between GD patients and healthy controls, (ii) to evaluate the balance shift in Th1/Th2 cytokines in patients with or without ophthalmopathy and (iii) the assessment in the relationship between mentioned cytokines and disease severity.

MATERIALS AND METHODS

Sera of 34 patients with GD (17 women, 17 men, mean age 35±13 years) were collected in endocrinology laboratory. The diagnosis of disease was based on clinical manifestations and chemical laboratory results (thyroid-stimulating hormone (TSH), free T4, free T3, thyroid autoantibodies, including TRAb, TPOAb and TgAb).

GO was evaluated by eye examination performed by an ophthalmologist no more than one month after the diagnosis. Ophthalmopathy activity was assessed according to the NOSPECS classification and was divided into three overall grades: mild, moderate and severe (Table 1).[10] Sera from 33 healthy individuals (17 women, 16 men, mean age 32±8 years), with no history of either GD or autoimmune disorders were used as controls. Table 2 presents the features and laboratory results of patients with GD and controls.

The serum concentrations of IL-2 (kit from Stressgen, Ann Arbor, Michigan), IFN-γ (kit from Stressgen, Ann Arbor, Michigan), IL-4 (kit from Duoset IC; R&D Systems, Minneapolis, Minnesota) and IL-10 (kit from Stressgen, Ann Arbor, Michigan) were quantified by ELISA. Assay sensitivities were 6.6 pg/ml for IL-2, less than 2 pg/ml for IFN-γ, less than 10 pg/ml for IL-4 and 4 pg/ml for IL-10. All serum samples of patients and controls were tested for antibodies directed against thyroglobulin, thyroid peroxidase and TSH receptor by ELISA kit (DRG International Inc., USA). The lower detection limit for TRAb, TPOAb, and TgAb were 0.21 U/L, 5 IU/mL, and 10 IU/mL, respectively.

Table 2. The features and laboratory results of patients with graves' disease and controls

Topics	Patients	Controls	Statistical significance
Age (years)	35±13	32±8	NS
Sex (female/male)	17/17	17/16	
Thyroid stimulating hormone receptor antibody (U/L)	19.4±2.6	1.5±1.1	S
Thyroid peroxidase antibody (IU/mL)	159±38	14.1±1.6	S
Thyroglobulin antibody (IU/mL)	312±116	33.4±17	S
Interleukin-2 (pg/ml)	8.5±1	7.8±0.7	S
Interferon-γ (pg/ml)	11.5±5.6	17.1±0.7	S
Interleukin-4 (pg/ml)	24±5.6	20±5	NS
Interleukin-10 (pg/ml)	9.9±2.4	6.7±2.9	S
Thyroid-Stimulating Hormone (mIU/L)	0.7±0.3	2.1±0.3	S
Free T4 (ng/dL)	201±101	1.2±0.2	S
Free T3 (pg/ml)	7±0.9	3.3±0.06	S

NS=not significant, S=significant

Statistical Analysis

Hormone and cytokine levels were compared between groups using Student's t test. Mann-Whitney U test was applied for analysis of difference in Th1/Th2 cytokine ratio of patients with GD and controls. Correlations between various parameters were calculated by Pearson test. All data are presented as mean±SD. *P*-values lower than 0.05 were considered statistically significant.

RESULTS

The mean duration of GD was 34±8 months. The diagnosis of GD was confirmed by measurement of serum levels of free T4, free T3, TSH and thyroid autoantibodies. Circulating TSH levels were significantly lower and T3 and T4 levels were significantly higher in patients with GD compared to the control group. In addition, the titers of TRAb, TPOAb and TgAb were significantly increased (p=0.001) in patients compared to controls (Table 2).

The serum cytokine profiles were evaluated in both GD patients and control individuals. Statistical analysis of these cytokines revealed that levels of IL-2 (*p*=0.004) and IL-10 (*p*=0.001) were significantly elevated (Figure 1) in GD patients compared to controls. The mean values of IL-2 and IL-10 were 8.5±1 pg/ml and 9.9±2.4 pg/ml, for patients and 7.8±0.7 pg/ml and 6.7±2.9 pg/ml for controls, respectively.

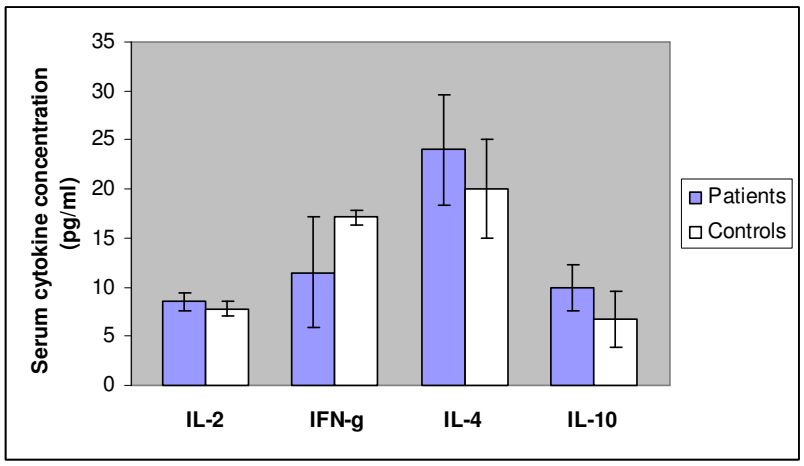

Figure 1. Serum cytokine levels of GD patients and controls

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Table 3. Th1/Th2 cytokine ratio of patients with graves' disease and controls

Group	IL-4/IL-2	IL-4/IFN-γ	IL-10/IL-2	IL-10/IFN-γ
Patients	2.8±0.8	3.7±0.7	1.3±0.1	2.3±0.2
Controls	1.6±0.3	0.8±0.1	0.9±0.1	0.6±0.1
P value	NS	S	S	S

NS=not significant, S=significant

Conversely, the mean concentration of IFN-γ was significantly higher in control individuals than patients (p=0.001). Moreover, there were no significant differences between groups with regard to the levels of IL-4. Cytokine levels were also compared in patients with or without ophthalmopathy and no significant differences were observed between cases and control subjects. When the patients with ophthalmopathy were categorized into three groups: severe (N=5), moderate (N=8) and mild (N=5) according to the NOSPECS classification, similar cytokines profiles were observed in all sub-groups. Nonetheless, serum IL-10 level was significantly higher in patients with moderate and severe ophthalmopathy than those without ophthalmopathy. In addition, the ratio of serum cytokines IL-4/IFN-γ, IL-10/IL-2 and IL-10/IFN-γ increased in patients with GD in comparison to matched controls (Table 3).

In another part of the study, the correlation between the levels of cytokines and disease severity was analyzed. It was found out that there is no relationship between serum cytokine levels (IL-2, IFN- γ, IL-10 and IL-4) and disease severity in this research samples.

Moreover, no associations were observed between serum levels of cytokines and duration of the disease. The only correlation that was detected was between serum TRAb concentrations and serum levels of free T3 (r=0.39, p<0.001) and free T4 (r=0.4, p<0.001).

DISCUSSION

GD is the prototype autoimmune receptor disease mediated by autoantibodies against the thyrotropin receptor. It may be associated with an inflammatory process in the orbit recognized as GO. The etiology of GD is not well understood and it appears different causative factors implicate in the pathogenesis of disease.[11]

Among these factors, autoimmunity has a high possibility of causing GD. Skewing of immune responses may arise as a result of break down in self-tolerance, which produces abnormal thyroid-immune interactions, implicating an array of cytokines and their receptors.[12]

GD is generally considered to be a Th2-dominant disease. Cytokines such as IL-4, IL-5, IL-10, and IL-13, are secreted by intrathyroidal lymphocytes, all of which tend to support antibody mediated immune responses.[13] However, Nagayama et al. indicated that an immune response shifting toward Th2 was accompanied by a decline rather than an increase in synthesis of thyroid stimulating antibody, which also propose that major Th1 immune responses to TSH receptor are associated with induction of GD.[14] Moreover, human TSH receptor stimulating antibodies are mainly IgG1, a Th1 type subclass in humans.[15] Therefore, it is not clear whether GD is a Th1- and/or Th2-mediated disease.

Cytokines also play a key role in the development of GO. This form of the disorder is characterized by edema and inflammation of the extraocular muscles and an increase in orbital connective tissue and fat.[16] It is believed that lymphocytes and mast cells migrate to the involved tissues and that cytokines produced by these cells contribute to tissue reactivity and remodeling.[17-19]

To date, there is controversy about the phenotypic characteristics of T lymphocytes that predominate during the active phase of GO. Several studies have analyzed the cellular infiltration in GO. The results of these investigations indicate that, Th1 and Th2 cells accumulate in connective/adipose tissue and extraocular muscles.[17,19] A wide range of Th1 and Th2 cytokines has been detected in affected orbital tissues.[20,21] For instance, mRNA encoding TNF-α, IL-1β, IFN-γ, IL-4, IL-6, and IL-10 was detected in muscle and orbital fat.[21] In addition, immunohistochemical studies indicated the presence of cytokines, including IFN-γ, TNF-α, and IL-1α in the connective tissues, and suggested their existence is associated with T cell infiltration.[22] GD is also associated with elevations in cytokine levels in serum.

Nonetheless, studies on the changes of serum cytokines in GD frequently provide conflicting results.[23-25]

The concept of association between GD and IL-2 formed on the basis of several important observations. For example, there is a significant rise in the number of B cells capable to bind IL-2; this was particularly apparent in untreated hyperthyroid GD patients. In addition, a greater number of CD251 (IL-2R subunit p55/a chain) T and B cells were found in hyperthyroid untreated GD patients than in long-term remission euthyroid patients. Moreover, several studies indicate that patients with active GO have increased levels of IL-2, IL-6, sIL-2R, and sIL-6R as compared to control subjects.[26,27]

Consequently, increased production of IL-2 can be interpreted as the activation of the immune system and as an indication of IL-2 significance in the pathogenesis of this syndrome.

The role of IFN-γ in the pathogenesis of GD is controversial. On the other hand, anti-IFN-γ neutralizing antibodies could prevent the disease and reduce Tg-specific T cell responses.[28] In addition, it has been shown that thyroid infiltrating lymphocytes produce high levels of IFN-γ and facilitate apoptosis of thyroid follicular cells via caspase activation.[29] Furthermore, some studies have shown that IFN-γ plays a dispensable role in the induction of autoimmune thyroid disease. For instance, IFN-γ and IFN-γ receptor knockout mice exhibit susceptibility to experimental autoimmune thyroiditis (EAT).[30,31] Moreover, it has been reported that IFN-γ suppresses EAT.[32] In our study, we determined that the levels of IFN-γ decreased in GD patients compared with control subjects. These findings are in agreement with several other studies demonstrating defective IFN-γ production by peripheral blood mononuclear cells (PBMCs) in GD.[33,34]

While the exact reason for this phenomenon is unclear, a number of possible explanations can be offered. For instance, high levels of IL-2 may downregulate IFN-γ production in GD patients. This effect may be due to a reduction in CD4 cells in response to IL-2 stimulation in GD. Furthermore, there may be alterations in the number and function of IL-2R on CD4 cells in GD thereby may abrogate IL-2 induced activation of PBMCs. Another possibility could be related to defect of intra-CD4 cellular IL-2 signal transduction resulting in the IFN- γ production or release in GD.[34,35]

The present study also indicates substantial increase in the production of IL-10 which is similar to well documented observations described previously by Takeoka et al.[36] The importance of IL-10 in GD was highlighted by recent investigations. These studies showed elevated levels of serum IL-10 and IgG3-secreting cells in patients with intractable GD, but not in GD patients during remission or in Hashimoto's thyroiditis patients.[36,37] IL-10 can upregulate the synthesis of the complement-activating isotypes IgG1 and IgG3. Therefore, it may support the formation of complement opsonized complexes between self-antigens and autoantibodies, and the subsequent uptake and presentation of self-antigens by B cells.[38,39] Type 1 regulatory T (Tr1) cells are the main IL-10 producers for suppression of autoimmune processes.[40] Moreover, B cells have been shown to be able to provide IL-10-mediated protection against autoimmunity in mice.[41] These findings are in accordance with a number of existing research that indicate IL-10 plays a protective role in several animal models of autoimmune diseases[42,43], probably by inhibiting Th1 cytokine responses together with IL-4.[42-44] In contrast to the above studies, there are reports showing that IL-10 exacerbates antibody-mediated autoimmune diseases such as systemic lupus erythematosus and myasthenia gravis.[45,46] This cytokine plus IL-4 are Th2 cytokines that preferentially stimulates humoral rather than cellular immunity, although, the role of IL-4 in these diseases is not apparent.[47] Therefore, it seems unclear whether IL-10 production signifies a response of Tr1 cells and/or B cells to control autoimmune processes, or whether it promotes pathogenic processes.

Altogether, these data highlight, once again, that cytokine actions are the result of a complex network, and further investigation in this field is expected to reveal the exact mechanism(s) by which these cytokines mount their detrimental or beneficial effects.

In another part of our study cytokine levels were compared in patients with or without ophthalmopathy. The results obtained indicate no significant difference in the concentrations of these cytokines between these two groups.

The findings were also analysed in relation to the severity of ophthalmopathy. The results indicated that serum IL-10 level was significantly higher in patients with moderate and severe ophthalmopathy than those without ophthalmopathy. In the next step, the relation between serum cytokine levels and TRAb, TPOAb and

TgAb concentration was investigated and no correlation was observed between serum levels of antithyroid antibodies or thyroid hormones and serum levels of cytokines.

It must be mentioned that there were also some limitations in this investigation. The sample size was relatively small, which did not allow us to determine the potential differences between the GD patients with and without ophthalmopathy. Additionally, in this study we assessed the levels of only four cytokines. While other cytokines, chemokines and growth factors which were not determined, may also play a significant role in the pathogenesis of the disease.[48]

Overall, this study indicated elevated serum concentrations of thyroid autoantibodies together with IL-10. Increased ratio of Th2/Th1 cytokines from GD patients may reflect the importance of humoral immunity. Moreover, significantly higher levels of IL-10 were detected in patients with moderate and severe ophthalmopathy than those without ophthalmopathy. Therefore, this cytokine may contribute to the immunopathology observed in ocular complications associated with thyroid dysfunction. Clearly, more studies are required to further shed light to the T-cell types and their cytokines involved in the pathogenesis of GO patients.

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