Serum cytokines levels in Graves' disease

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ABSTRACT

Objective: The aim of the study was to look at the serum cytokines profile in newly diagnosed thyrotoxic patients with Graves' disease and to compare their cytokine levels with those of normal control subjects. Furthermore, the levels of T4, being an indicator of the severity of thyrotoxicosis, were also correlated with the Th1/Th2 and proinflammatory cytokines in Graves' disease patients.

Methods: Serum IFN-γ (Th1), IL-10 (Th2), inflammatory cytokines including IL-6, TNF-α, sCD23 and sIL-2R cytokine levels were measured in 28 patients with Graves' thyrotoxicosis and in 30 normal controls.

Results: In Graves' disease patients, the levels of IFN-γ (mean 142.1 ± 29.53 units/ml), IL-10 (mean 583.8 ± 253.3 pg/ml) and IL-4 (mean 132.4 ± 44.52 pg/ml) were significantly higher than their corresponding levels in controls: IFN-γ (mean 31.6 ± 2.08 units/ml, P<0.001), IL-10 (mean 69.8 ± 31.72 pg/ml, P<0.001) and IL-4 (mean 46.44 ± 11.53 pg/ml). There was a marked increase in proinflammatory cytokines in Graves' disease patients: levels of IL-6 (481.5 ± 192.3 pg/ml) and TNF-α (30.69 ± 16.7 pg/ml) were significantly higher than those of normal controls for IL-6 (63.81 ± 21.72 pg/ml, P<0.001) and TNF-α (8.81 ± 1.72 pg/ml, P<0.001). Similarly the levels of sCD23 (mean 164 ± 67.03 ng/ml) and sIL-2R (mean 2131 ± 461.1 units/ml) were significantly higher in GD patients than in the control group (mean 31.24 ± 11.53 ng/ml, P<0.001) and (mean 345.53 ± 121.75 units/ml, P< 0.001) for sCD23 and sIL-2R. Furthermore, in thyrotoxic Graves' disease patients, we detected a positive correlation between free T4 and sIL-2R levels (r^2 = 0.81, P<0.00), but no significant correlation was found between T4 and the other measured cytokines.

Conclusion: The elevated serum cytokines of Graves' thyrotoxic patients reflect the activation and interplay of mixed Th1 and Th2 cells which may be consistent with long standing inflammatory and destructive processes of thyroid gland. The clinical severity of hyperthyroidism in Graves' patients only correlated with sIL-2R.

Keywords: Graves' disease, serum cytokines.

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Graves' disease (GD) is an autoimmune disorder manifested clinically by features of hyperthyroidism, infiltrative ophthalmopathy and dermopathy. Infiltration of immune cells is a characteristic feature of thyroid gland of patients suffering from GD.[1] Immunohistochemistry, reverse transcription-polymerase chain reaction (RT-PCR) and protein staining by flow cytometry, demonstrated that intrathyroidal T lymphocytes produce interferonγ (IFN-γ) IL-2, IL-4 and IL-10. Immune regulatory cytokines induce B-cell differentiation that led to the production of anti thyroid antibodies; TSH receptor antibodies (TRAb), anti-thyroglobulin (TG) and anti-proxidase (TPO).[2-6] Macrophages and other related cells release, during the immune response, proinflammatory cytokines such as IL-1, IL-6, IL-8, IL-10, IL-12 and tumor necrosis factor (TNF).[5-10] Thyroid follicular cells (TFCs) are capable of producing an array of cytokines under normal conditions and in GD, such as IL-1a, IL-6, IL-8, IL-12, IL-13, and IL-15.[5,6,9] Those cytokines are water-soluble polypeptides which like hormones can have autocrine and paracrine effects. These enable the immune cells to communicate and play an integral

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role in the initiation, perpetuation, and subsequent down regulation of the immune response. Furthermore, cytokines have direct effect in TFCs by inducing expression of HLA class II molecules, and modulating growth, iodide uptake, and the production of anti thyroid antibodies. These cellular immune events of the activated and infiltrated thyroid tissue project in the concentration of cytokines in the peripheral blood circulation.[7-9]

The aim of the present study was to look at the serum cytokines profile in newly diagnosed thyrotoxic patients with GD and to compare them with normal control subjects, and to correlate the levels of Th1/Th2 and proinflammatory cytokines with the T4 levels as an indicators of the severity of thyrotoxicosis in GD patients.

Methods. Group 1 included 28 patients with hyperthyroid Grave's disease; 22 females and 6 males with mean age of 28 $\pm$ 2.5 years. Their diagnosis was based on the presence of clinical features of hyperthyroidism, exophthalmos and diffuse small goiter with the mean free T4 42 $\pm$ 1.2 pmol/L and TSH <0.0l mIU/L.

Group 2 included 30 normal controls, 20 females and 10 males with mean age of 35 + 3.6 years. These were clinically euthyroid with normal thyroid gland examination. The mean free T4 for this group was 16.2 pmol/L and TSH level of 3.2 mIU/L.

Cytokines measurement. IFN-γ Assay. IFN-γ was determined using sensitive and specific enzyme linked immunosorbent assay (ELISA). In brief, 96-well micrometer plates (25806 Corning, New York, NY) were coated overnight at 4ºC with 100 ul (diluted 1:800 in 0.1m carbonate, Ph9.5) of anti-human IFN-γ (80-3959-02, Genzyme, Cambridge, MA). Wells were washed five times with phosphate-buffered saline (PBS) pH7.3 with 0.05% Tween 20. The wells were blocked with 250 ul of 4% bovine serum albumin (BSA) in PBS, pH7.3 for 2 hours at 37ºC and 100 mL of serum samples and IFN-γ standards (25-750 unit/ml) were added to wells and incubated at 37ºC for 2 hours, plates were washed five times with PBS-0.05% Tween and 100 ul of second antibody, anti-human IFN-γ-HRP conjugate (2.5 ug/ml) was added to wells and incubated for 30 minutes at 37ºC. The wells were washed five times with PBS-0.05% Tween and 100 ul of substrate reagent tetramethylbenzidine (KPL, USA) was added to all wells for 30 minutes at room temperature. The reaction was stopped by adding 100 ul of $2NH_2SO_4$ and the optical density was measured at 450nm using a flow titertek microplate reader. The sensitivity of the IFN-γ test is 25 unit/ml.

IL-10 Assay. IL-10 was determined using IL-10 specific monoclonal (mAbs) in microtiter plates. In brief, the above mentioned micrometer plates were coated overnight at 4ºC with 2 ug/well (100 ul) of anti-human IL-10 mAb (#18551D, PharMingen, San Diego, CA). Wells were blocked with 1% bovine serum albumin (BSA)/phosphate buffered saline (PBS)-0.1% Tween, overnight at 4ºC. After five washes with PBS-0.1% Tween, standard (human IL-10) and serum samples were added to wells and allowed to incubate overnight at 4ºC. Plates were then washed five times with PBS-0.1% Tween and secondary detection mAb, biotinylated anti-IL-10 (# 18562D, PharMingen), 1 ug/ml (100 ul) in 10% BSA/PBS was added to each well. After 60 minutes of incubation at room temperature, plates were washed five times (PBS-0.1% Tween) and 100 ul of peroxidase-conjugated streptavidin, diluted 1: 1000 in 10% BSA/PBS was added to each well and allowed to incubate for an additional 60 minutes at room temperature. Plates were washed five times (PBS-01.% Tween), after which bound secondary mAb was allowed to react with substrate (100 ul/ well, 3,3', 5,5' tetramethylbenzadine; Sigma) for 10 minutes. Reaction was stopped by adding 2 mol/L H_2SO_4 (50 ul/well) and optical density was determined in a Flow Titertek microplate reader at 450 nm. The sensitivity of IL-10 in this ELISA was 40 pg/ml.

IL-4 Assay. IL-4 levels was measured in serum samples by a solid phase ELISA based on the antibody Sandwich principle, using an Interest ELISA kit (code number IT-4) purchased from Genzyme (Boston, MA, USA). The immunoassay was performed as follows: Microtiter plates (25806 corning, New York, NY) were coated using 100 ul of anti IL-4 monoclonal antibody (1:125) and incubated at 4ºC overnight. The plates were washed 4 times with wash buffar and 100 ul of serum samples and standards (45-3000 pg/ml) were added and incubated for 2 hours at room temperature. After that the plates were washed 4 times with washing buffer and 100 ul of ready to use rabbit anti-IL-4 antibody was added to each well and incubated for 2 hours at room temperature. The plates were washed 4 times with washing buffar and 100ul of biotinylated goat anti-rabbit immunoglobulin antibody (1:6500) was added to all wells and incubated at room temperature for 45 minutes then washed 4 times with the wash buffer and 100 ul of streptaridin-conjugated horse radish peroxidase (1:7000) was added to each well and incubated for 40 minutes at room temperature followed by plate washing for 4 times and 100 ul of the substrate reagent (0-phenylin diamine-peroxide) was added to all wells and incubated for 5-10 minutes. The reaction was stopped by adding 100ul of 1M H_2SO_4 to all wells. The plate was read at 492nm using a flow titertek microplate readar and IL-4 values were obtained from the standard cure. The sensitivity of the IL-4 test is 45 pg/ml.

IL-6 Assay. IL-6 levels were measured in serum samples by a solid phase ELISA based on the antibody sandwich principle, using an Intertest

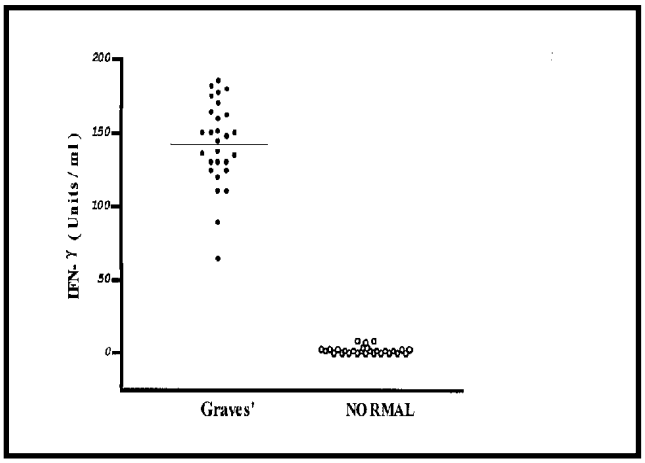

Figure 1 - Scatter plot of INF-y levels in Graves' and normal controls. The solid line represents the mean level of INF-y in each group.

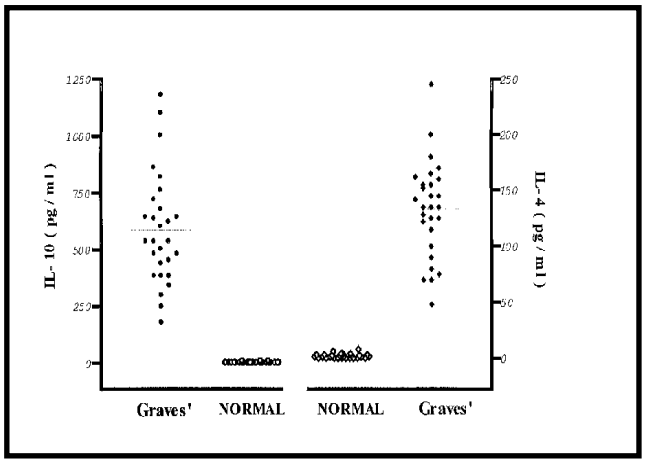

Figure 2 - Scatter plot of Il-10 (left Y-axis), and IL-4 (right Y-axis) levels in Graves', and normal controls. The solid line represents the mean level in each group.

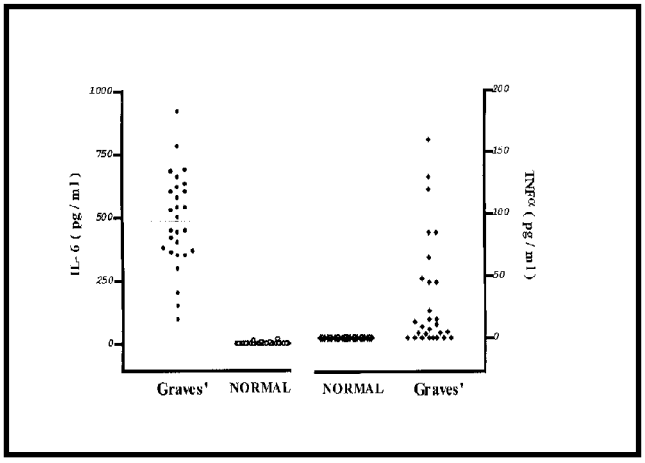

Figure 3 - Scatter plot of IL-6 (left Y-axis), and IL-TNFx (right Y-axis) levels in Graves', and normal controls. The solid line represents the mean level in each group.

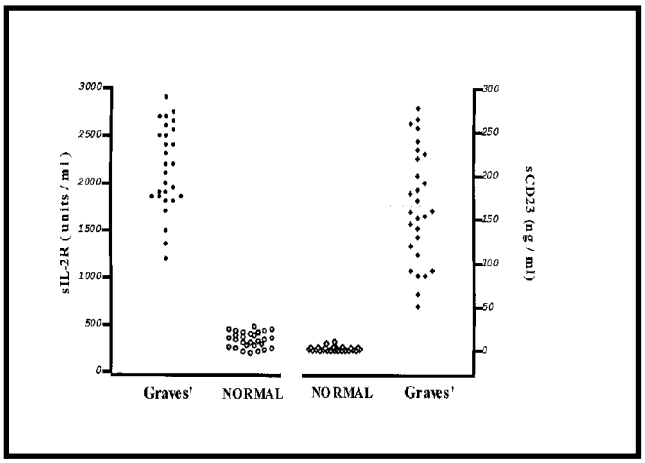

Figure 4 - Scatter plot of sIL-2R (left Y-axis), and SCD23 (right Y-axis) levels in Graves', and normal controls. The solid line represents the mean level in each group.

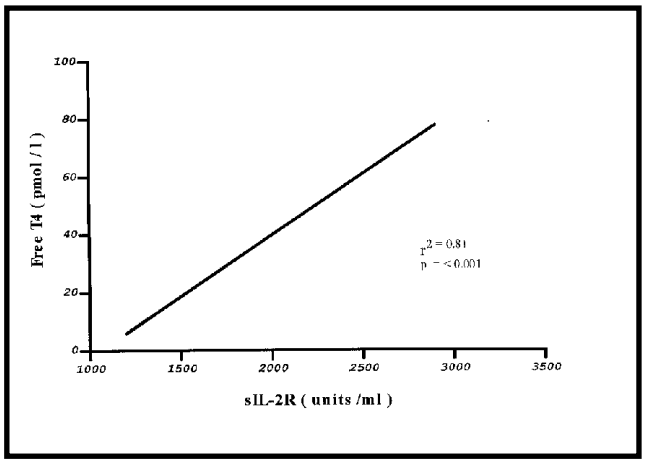

Figure 5 - Linear regression curve of free T4 upon sIL-2R among thyrotoxic Graves'patients.

ELISA kit purchased from Genzyme (Cambridge, MA. USA). The immunoassay was performed as follows: 100 ul of diluted serum samples (1:2) and standards (150 - 2500 pg/ml) were added to microtiter plates precoated with anti-IL-6 monoclonal antibody and incubated for 30 minutes at 37°C. The plates were washed 5 times with washing buffer and 100 ul of biotinylated polyclonal rabbit anti-IL-6 (ready to use) was added to all wells and incubated for 30 minutes at 37°C. The wells were washed 5 times with washing buffer and 100 ul of avidin-peroxidase conjugate (ready to use) was added to all wells and incubated for 15 minutes at 37°C. The wells were washed 5 times using washing buffer and 100 ul of substrate solution (tetrmethyl benzidine-mg hydrogen peroxide) was added to all wells and incubated for 8 minutes and the color was stopped by adding 100 ul of 1M sulfuric acid. The absorbance was read at 450 using a flow titertek microplate reader and IL-6 values were obtained through the standard curve. The sensitivity of the test was 150 pg/ml.

TNF-α Assay. TNF-α was measured by a standard enzyme linked immunosorbent assay (ELISA). In brief, wells of microtiter plates (Nunc, Immunotype II) were coated with a purified polyclonal antibody against recombinant human TNF-α (rhTNFα; Genzyme) for 2 hr, then blocked by incubation overnight with 2% bovine serum albumin. After washing, serial dilution of standard rhTNFα or samples were added (100ul/well) and the plates were incubated at room temperature for 2 hours. After further washing, purified monoclonal antibody against rhTNFα biotinylated sheep antibody against mouse Ig, and streptavidin biotinylated horseradish peroxide complex were added. The yellow color developed with orthophnyline diamine substrate was read at 450 nm in a Titertek Multiskan ELISA plate reader. The limit of the assay was 10 pg/ml.

Soluble CD23 (sCD23) assay. The levels of sCD23 in-patients sera were determined using a commercial kit (Binding site U.K) based on the sandwich enzyme immunoassay (EIA). Some 100 ul of patient's sera and standards was added to coated microtitre plates and incubated for two hours at 37°C. After that plates were washed three times using washing buffer and 100 of diluted anti sCD23 (1:200) was added to each well and incubated at 37°C for two hours. At the end of the incubation period, the plates were washed three times and 100ul of diluted anti-sheep IgG conjugated to horseradish peroxidase and incubated at 37°C for two hours, then plates were washed three times and 100ul of o-phenylenediamine substrate was added and incubated for 30 minutes at room temperature, the reaction was terminated by adding 50 ul of stopping solution and the optical densities of each well were read at 450nm. Results were obtained through a standard curve, the sensitivity of the assay is 6ng/ml.

Soluble IL-2 receptor (sIL-2R) assay. sIL-2R were measured with an enzyme immunoassay with commercially available kits (T cell Sciences, Cambridge, MA). Intraassay and interassay coefficients of variation were 4% and 7%. Serum sIL-2R concentration were expressed in unit/ml; 1000 U is defined as the amount of released sIL-2R present in 1.0 ml of a reference preparation of supernatant from PHP-stimulated blood lymphocytes. The normal values obtained for the control group were less than 480 u/ml.

Statistical analysis. Experimental data were entered in a computer. Scattergrams and statistical analysis were performed by PrismTM version 2 (GraphPad software, Inc, San Diego, USA) and SPSS, Student t-test was used to compare the differences in mean values between groups, multiple regression analysis was used to determine the direct relationship of free T4 to serum cytokines level.

Results. Th1/Th2 serum cytokines patterns were measured and compared in both groups. These included the measurement of INF-γ, IL-10 and IL-4 level. The mean level of INF-γ, IL-10 and IL-4 were elevated in GD patients and were significantly higher in GD patients than normal controls.

IFN-γ in GD patient (mean 142.1 $\pm$ 29.53 unit/ml), was significantly higher than normal control (mean 31.6 $\pm$ 2.08, $P < 0.001$) (Figure 1). IL-10 in GD patients (mean 583.8 $\pm$ 253.3 pg/ml) was significantly higher than normal controls (mean 69.8 $\pm$ 31.72, $P < 0.001$) (Figure 2). IL-4 in GD patients (mean 132.4 $\pm$ 44.52 pg/ml) was significantly higher than normal controls (mean 26.44 $\pm$ 11.43, $P < 0.001$) (Figure 2).

There was a marked increase in inflammatory cytokines including IL-6 and TNF-α. IL-6 in GD patients (mean 481.5 $\pm$ 193.2 pg/ml) was significantly higher than in normal controls (mean 63.81 $\pm$ 21.72, $P < 0.001$) (Figure 3). TNF-α in GD (mean 30.69 + 16.7 pg/ml) was significantly higher than in normal controls (mean 8.81 $\pm$ 1.72, $P < 0.001$) (Figure 3).

We also measured sCD23 and sIL-2R in GD and compared them to normal controls. sCD23 in GD patients (mean 164.9 $\pm$ 67.03 ng/ml) was significantly higher than normal controls (mean 31.42 $\pm$ 11.53 , $P < 0.001$) (Figure 4). sIL-2R in GD patients (mean 2131 $\pm$ 461.1 unit/ml) was significantly higher than normal controls (mean 345.53 $\pm$ 121.75, $P < 0.001$) (Figure 4).

The level of T4 in GD patients was correlated with the cytokines levels. A positive correlation was

detected between free T4 and sIL-2R level (r2 =0.81, p = <0.001) but no correlation was found between T4 and the other measured cytokines, (Figure 5).

Discussion. Serum Th1 (IFN-γ, Th2 (IL-10 & IL-4), inflammatory (IL-6, TNFα), sCD23 cytokins and IL-2R levels were measured in Saudi patients with Graves' disease (GD) in comparison to normal Saudi controls. It's apparent that mixed Th1 and Th2 related serum cytokines were significantly elevated in Graves' patients. This pattern was consistent with the mixed Th1/Th2 lymphocytes activity seen in the thyroid gland of GD patients which may have been involved in specific TSH-R antibodies production and cell mediated tissue destruction.[6] Also this observation is in agreement with the analysis of intrathyroidal cytokine gene expression profiles which showed the presence of both types of Th1 (INF-γ) and Th2 (IL-10 & IL-4) genes in the thyroid gland cells of patients with Graves' hyperthyroidism with a predominance of Th1 CD4 helper cells.[3-6] However, the two types of T-helper cells and associated cytokines usually interact with each other and that doesn't necessary mean the predominant result is cytotoxicity.

The inflammatory cytokines (IL-6, TNFα) were clearly elevated in Graves' disease patients.[7,8] Both cytokines could be produced by intrathyroidal lymphocytes and thyroidal follicular cells. Serum IL-6 is increased in autoimmune thyroid disease, multinodular goiter and posts radioactive treatment and has been considered as a marker of thyroid destructive inflammatory processes,[9] it also declines after propylthiouracil treatment.[10] TNFα has an inhibitory role in thyroid metabolism by suppressing basal and TSH-stimulated iodide transport in TFCs.[11] It also has a stimulatory effect in the autoimmune process by induction of HLA class II antigen presenting cell interaction with T cell; this may explain the persistence of the elevation of its level even after restoration of euthyroid status by propylthiouracil.[10]

Serum sCD23 levels are significantly elevated in all Graves' patients compared to normal control individuals, this in agreement with reports by other studies which indicated an elevated levels of sCD23 in autoimmune thyroidites, rheumatoid arthritis, systemic lupus erythematosis and Sjogren's syndrome.[12,13] Regulation of cell surface CD23 is maintained by the opposing action of IL-4 and INF-γ: IL-4 up-regulate while INF-γ down-regulates cell surface CD23 levels; so the interplay between Th1 (INF-γ) and Th2 (IL-4) cells in the thyroid gland of patients with Graves' hyperthyroidism will determine the degree of sCD23 elevation.[13]

The serum level of sIL-2R was increased in Graves' patients compared to normal individuals, confirming previous observation of increase sIL-2R levels in autoimmune thyroiditis.[13] Also sIL-2R was shown to be elevated in non autoimmune type of thyrotoxicoisis such as toxic nodular goiter and toxic adenoma.[14-16]

The clinical manifestations seen in GD are due to elevated thyroxin levels. We looked at the correlation between all tested cytokines and T4 level. We observed significant correlation only with serum sIL-2R level (r2 = 0.81, P =<0.001). This observation has been noted in thyrotoxic patient independent from the etiology of their hyperthyroidism and the level of sIL-2R decreased after treatment with methimazole and surgical resection of Graves' disease.[15-18] These findings may suggest an association between hyperthyroxinemia and activation of human lymphocytes in thyroid gland.

Measurement of cytokine level could be an indicator of prognosis and outcome of treatment. However long-term follow up of cytokine levels in patient attending treatment clinics over an extended period of time may be required to establish their prognostic value. The elevated serum cytokines of Graves' thyrotoxic patient reflect the activation and interplay of mixed Th1 and Th2 cells which may be consistent with long standing inflammatory and destructive process of thyroid gland. The clinical severity of hyperthyroidism in Graves' patients is only correlated with sIL-2R.

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