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Analysis of peripheral blood T-cell subsets, natural killer cells and serum levels of cytokines in children with Down syndrome

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Summary

The objective of this study was to evaluate the relationship between humoral and cell-mediated immune response parameters and impairment of immune functions in children with Down syndrome (DS). The patient group was consisted of cytogenetically documented 32 children with DS. Lymphocyte subsets and natural killer cells were counted by flow-cytometry system. Levels of interleukin (IL)-1β, IL-2, IL-4, IL-6, IL-8, IL-10 and tumour necrosis factor-alpha (TNF-α) were detected by enzyme-linked immunosorbent assay method. Serum IgG, IgM, IgA levels were measured by turbidimetric methods. The percentage of CD8+ lymphocytes and CD56+ cells of patients with DS were significantly higher, whereas CD20+ lymphocytes were lower than that of controls ($P < 0.05$). The percentage of CD2 and CD4 levels and CD4/CD8 ratio of patients with DS and normal controls were similar ($P > 0.05$). Levels of IL-4 and IL-10 were significantly increased, but IL-6 and TNF-α levels were decreased in children with DS ($P < 0.05$). Levels of other studied cytokines between patients with DS and controls were not statistically different ($P > 0.05$, for all). Serum IgG, IgM and IgA levels were found to be similar between the groups ($P > 0.05$). It has been known that IL-4 and IL-10 are anti-inflammatory molecules which inhibit the synthesis of proinflammatory cytokines such as IL-6 and TNF-α. In this study, levels of IL-4 and IL-10 were significantly increased, but IL-6 and TNF-α levels were decreased in children with DS. These results may suggest that continuing anti-inflammatory state in DS and this process may explain the cause of recurrent infection of the disease. On the other hand, in contrast to the low percentage of CD20+ cells, high percentage of CD8+ and CD56+ cells were found. Our findings may demonstrate that the cell-mediated and humoral immune system parameters in children with DS were altered according to healthy children.

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Introduction

Down syndrome (DS) is a chromosomal alteration characterized by the presence of an extra copy of the chromosome 21. It is well known that subjects with DS have an increased susceptibility to bacterial and viral infections, malignancies, and autoimmune disorders and these situations are the main cause of mortality and morbidity in most of the cases (Karttunen *et al.*, 1984; Raziuddin & Elawad, 1990; Nespoli *et al.*, 1993; Park *et al.*, 2000; De Hingh *et al.*, 2005). The number of circulating T and B cells has been shown to be abnormally low in patients with DS and their lymphocyte responses against T- and B-cell mitogens are weak. In addition, reduced levels of CD4+ T lymphocytes (T helper/inducer) and increased levels of CD8+ T lymphocytes (T suppressor/cytotoxic) as compared with healthy subjects were also reported (Karttunen *et al.*, 1984; Colombo *et al.*, 1989; Cossarizza *et al.*, 1990; Raziuddin & Elawad, 1990; Ugazio *et al.*, 1990; Nespoli *et al.*, 1993; Licastro *et al.*, 2001).Some reports demonstrate an increase in circulating levels of natural killers (NK) in patients with DS whereas others indicate a decrease; however, they all emphasize that the appearance of functionally defective cells. Humoral immune-deficiencies were also reported in DS. In most of these reports, the number of B lymphocytes was found to be decreased and their ability to produce antibodies was diminished. The interpretation of serum immunoglobin levels is rather difficult because of the variety of reported changes. There is a tendency in the literature for higher IgG levels, accompanied with a reduction of IgG2 and IgG4, but an increase of IgG1 and IgG3 subgroups (McMillan *et al.*, 1975; Reiser *et al.*, 1976; Singh & Fudenberg, 1989; Cossarizza *et al.*, 1990; Bilgin *et al.*, 1996).

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Cytokines are soluble mediators produced during infection or inflammatory responses. Some cytokines such as interleukin IL-1, IL-6 and tumour necrosis factor-alpha (TNF-α) have proinflammatory effects that enhance inflammation and they are mainly produced by macrophages, T and B cells, and stimulate T- and B-cell immune responses. IL-1 potentiates the synthesis of IL-2. IL-6 is mainly involved in B-cell differentiation into IgG-producing plasma cells, and TNF-α is a major mediator of infection and induces chemotaxis and phagocytic activity of leucocytes. On the other hand, IL-4 and IL-10 have anti-inflammatory effects which are known to inhibit the production of proinflammatory cytokines (Steinbeck & Roth, 1989; Abbas & Lichtman, 2005).

In this study, we aimed to investigate some of the cell-mediated and humoral markers of 32 children with DS and age-matched control group were compared as to reveal the relationship between the changes of serum levels of such markers and immune impairment.

Materials and methods

Subjects

The patient group was consisted of cytogenetically documented 32 children with DS (14 males and 18 females, mean age ± SD: 3.9 ± 2.3, range 1–7 years) by the Department of Medical Biology and Genetics, Faculty of Medicine, University of Cukurova between June 2004 and March 2006 and 32 healthy children (18 males and 14 females, mean age ± SD: 4.5 ± 1.9, range 1–7 years) were included in the study. They were obtained from the same ethnic groups, and live in same geographic areas as the patients. The study was approved by the institutional review board and written informed consent was taken from all parents of the studied subjects. The study was performed in compliance with the declaration of Helsinki.

Quantitative detection of cytokines and immunoglobulins

Five millilitres of venous blood was drawn from all the subjects and the sera were immediately separated and aliquotted into two separate test tubes (75 × 12 mm polystyrene tubes). The aliquots were stored frozen at −80 °C until tested. At the day of analysis, the frozen samples were thawed, vortexed, and brought into room temperature. One of the aliquots was used to determine the cytokine levels while the other for the measurement of the immunoglobulins. Serum cytokines, namely, TNF-α, IL-1β, IL-2, IL-4, IL-6, IL-8 and IL-10 were measured by enzyme-linked immunosorbent assay (ELISA) using a commercially available high sensitive ELISA kit according to manufacturer's instructions (Immunotech; Beckman Coulter Company, USA). IgG, IgA and IgM levels were measured by turbidimetric assay by fully automatic Cobas Integra-800 system (Roche, Germany).

Determination of lymphocyte subsets and NK cells

The range of CD2, CD4, CD8, CD20, CD56 and the CD4/CD8 ratio in children with DS and healthy controls were determined by flow cytometry (Epics XL-MCL; Beckman Coulter). The flowing fluorochrome-conjugated monoclonal antibodies T11-RD1/B1-FITC, T4-RD1/T8-FITC (Beckman Coulter) and CD3-FITC/CD56-RD1 were used in this study.

Two millilitres of peripheral venous blood was collected into vacutainer glass tubes containing EDTA from all the subjects including the control group. The leucocyte number of each sample was resuspended at a concentration of minimum 6×10^6 cells mL^{-1}. For each parameter, 100 μL of blood was put into a 12 × 75 mm test tube and 10 μL of the appropriate fluorochrome-conjugated monoclonal antibody solution was added. The samples were incubated in the dark at room temperature for 20 min. Later, 600 μL of Immunoprep A (erythrocyte lysing agent), 265 μL of Immunoprep B (leucocyte stabilizer) and 100 μL of Immunoprep C (cell membrane fixative) solutions were added to the samples in the TQ-Prep Workstation (Beckman Coulter). Analysis of each lymphocyte subset was carried out using an Epics XL-MCL flow cytometer (Bekman Coulter) and the values were determined as a percentage of all the parameters. Absolute values of lymphocyte subsets (numbers of cells μL^{-1}) were calculated by the following formula: WBC × lymphocyte percentage × subset percentage (Kam *et al.*, 1996; Yaman *et al.*, 2005).

An initial fluorescence gating strategy was performed based on CD45+/CD14− back-gating and/or CD45+/side scatter gating.

For each analysis, 50 000 cells were counted. A range of internal quality assurance procedures was employed, including daily calibration of flow cytometer optical alignment and fluidic stability using FlowCheck (Beckman Coulter) fluorospheres and daily monitoring of whole blood preparation procedures and monoclonal antibody reactivity using ImmunoTrol (Beckman Coulter) control cells.

Statistical analysis

SPSS v15 (UK) was used for analysis of data. For normally distributed variables, the Student's *t*-test and for non-normally distributed variables the Mann–Whitney test was performed for comparison of cytokines, T-cell subsets, B cells and NK cells between groups. The chi-squared test was used to test for differences in gender between groups.

Results

The percentage of CD2 and CD4 levels of patients with DS and normal controls were similar ($P > 0.05$).

However, patients with DS had an increased percentages of, CD8+ lymphocytes and CD56+ cells (CD3−/CD56+) when compared controls whereas their CD20+ cells were decreased ($P < 0.001$, 0.001 and 0.05, respectively). The CD4/CD8 ratio of patients with DS and healthy controls were similar ($P > 0.05$) (Table 1). IL-4 and IL-10 levels were found to be significantly higher whereas IL-6 and TNF-α levels were significantly lower in patients with DS in respect to the control group ($P < 0.001$, <0.005, <0.001, <0.005, respectively) (Table 2). There was no statistically significant correlation between cytokine levels and the T-cell subsets ($P > 0.05$). There were no statistically significant differences between the groups for the other studied cytokines ($P > 0.05$). IgG, IgM and IgA levels showed no statistically significant differences between the groups ($P > 0.05$) (Table 3).

Table 1. The mean, standard deviation and *P*-values of percentages and absolute values of lymphocyte subgroups and natural killer cells of patients with Down syndrome and the control group

	Controls (mean ± SD)	Patients (mean ± SD)	*P*-value
CD2			
%	74.68 ± 1.7	79.35 ± 0.64	>0.05
Cell μL^{-1}	1.892 ± 137	1.801 ± 129	>0.05
CD4			
%	40.88 ± 1.4	43.03 ± 1.10	>0.05
Cell μL^{-1}	1.006 ± 75	1.114 ± 116	>0.05
CD8			
%	27.87 ± 1.57	3.31 ± 1.44	**<0.001**
Cell μL^{-1}	613 ± 55	753 ± 65	**<0.001**
CD20			
%	17.03 ± 1.72	12.65 ± 0.76	>0.05
Cell μL^{-1}	388 ± 54	222 ± 21	>0.05
CD56			
%	7.84 ± 0.14	14.35 ± 0.2	**<0.001**
Cell μL^{-1}	184 ± 15	310 ± 21	**<0.001**
CD4/CD8	1.55 ± 0.2	1.66 ± 0.1	>0.05

Bold values are statistically significant ($p \leq 0.05$).

Table 2. The mean, standard deviation and *P*-values of cytokine levels of patients with Down syndrome and the control group

($pg\ mL^{-1}$)	Controls (mean ± SD)	Patients (mean ± SD)	*P*-value
IL-1	11.68 ± 2.50	11.37 ± 1.82	>0.05
IL-2	12.68 ± 1.79	13.75 ± 2.44	>0.05
IL-4	81.75 ± 4.75	130.18 ± 20.81	**<0.001**
IL-6	23.75 ± 4.89	12.56 ± 1.89	**<0.001**
IL-8	27.06 ± 6.69	25.06 ± 3.83	>0.05
IL-10	384.62 ± 1.52	419.81 ± 67.61	**<0.005**
TNF-α	11.00 ± 1.50	9.62 ± 1.79	**<0.005**

IL, interleukin; TNF, tumour necrosis factor.
Bold values are statistically significant ($p \leq 0.05$).

Table 3. The mean, standard deviation and *P*-values of immunoglobulin levels of patients with Down syndrome and the control group

($mg\ dL^{-1}$)	Controls (mean ± SD)	Patients (mean ± SD)	*P*-value
IgG	1089.69 ± 165.69	1176.38 ± 203.12	>0.05
IgA	86.18 ± 16.50	87.87 ± 13.56	>0.05
IgM	88.56 ± 11.56	80.37 ± 33.96	>0.05

Discussion

Our study is designed to determine the relationship between abnormalities in immune function and cellular and humoral immune parameters in children with DS. It has been known that subjects with DS have an increased susceptibility to bacterial and viral infections, a high risk of malignancies, and autoimmune disorders according to healthy population, due to the impairment of the immune system (Castro *et al.*, 1993; Nespoli *et al.*, 1993; Scotese *et al.*, 1998). There are numerous studies in the literature of T lymphocytes and their subsets which is known to be the most important parameter of cell-mediated immune system in patients with DS (Colombo *et al.*, 1989; Cossarizza *et al.*, 1990; Ugazio *et al.*, 1990; Castro *et al.*, 1993; Nespoli *et al.*, 1993). In most of these studies, the total number of T cells have been reported to be slightly depressed or elevated, however, they all agreed on the impairment of T-cell functions (Karttunen *et al.*, 1984; Cossarizza *et al.*, 1990; Ugazio *et al.*, 1990; Nespoli *et al.*, 1993; Tolmie, 1997; Licastro *et al.*, 2001). Similar studies are performed for the T-lymphocyte subsets in DS and the data reported showed that an increase in the CD8+ T-cell subset was accompanied by a decrease in CD4+ T-cell subsets (Colombo *et al.*, 1989; Cossarizza *et al.*, 1990; Raziuddin & Elawad, 1990; Castro *et al.*, 1993; Nespoli *et al.*, 1993; Tolmie, 1997; Park *et al.*, 2000). We found a mild increase in CD4+ T-cell subset but it was not statistically significant. In this study, we observed a significant increase of the percentage of CD8+ subset. According to these findings, high percentage of CD8+ T lymphocytes may contribute to the state of recurrent infection in patients with DS stimulating the immune system. These results support the suggestion that continuing anti-inflammatory state in DS and this process may explain the cause of recurrent infection in these patients.

Although it was not statistically significant, a mild increase of CD4+/CD8+ ratio in children with DS compared to healthy controls was observed. These results were partially compatible with the findings of Colombo *et al.* (1989). All these findings suggest that the alterations of such parameters are not the same for all the patients, possibly because the T-helper cell function can be different in each patient with DS and led to a view that there is a functional change rather than the total number of CD4+ cell subsets. Rascon *et al.* (1984) suggested that these alterations of CD8+ T-cell subsets could be due to functional and structural alterations of the thymus. Colombo *et al.*

underlined the data of Benda & Strassmann (1965) that bioptic and autoptic material of children with DS showed hypoplasia of the thymus with poor histological differentiation between the cortex and medulla. Nespoli *et al.* (1993) reported that DS may be considered as a model of precocious, abnormal aging of the thymus-dependent immune system in man. According to our results, we considered that the impairment of the cell-mediated immune system of these patients may be due to the abnormal thymic development as well as the deficiency of the thymic endocrine activity needed for the maturation of T lymphocytes.

The results of T- and B-lymphocyte percentage were partially similar to the findings of De Hingh *et al.* In particular, we observed a mild increase in CD2+ T-lymphocyte percentage in patients with DS. However, CD20+ B-lymphocyte percentage was significantly decreased. Our results provide evidence that an increase in the percentage of the CD56+ cells occurs in the circulation of all children with DS compared to the control group, which is compatible with most of the reports in the literature (Cossarizza *et al.*, 1990; Ugazio *et al.*, 1990; Tolmie, 1997). One of the reasons of the susceptibility of patients with DS to viral infections and a high risk of malignancies was attributed to the observation of the appearance of functionally defective NK cells by most of the investigators (Cossarizza *et al.*, 1990; Ugazio *et al.*, 1990; Tolmie, 1997).

In our study, we investigated the serum levels of IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10 and TNF-α in children with DS and healthy controls. IL-4 and IL-10 levels were significantly higher in children with DS than in controls, whereas IL-6 and TNF-α levels were significantly reduced in DS. Levels of secretions of other cytokines in children with DS were not different from controls. IL-4 and IL-10 are anti-inflammatory mediators that inhibit the synthesis of a broad spectrum of proinflammatory cytokines such as IL-6 and TNF (Steinbeck & Roth, 1989; Abbas & Lichtman, 2005). The depressed levels of TNF-α and IL-6 in patients with DS possibly suggest impairment in the proliferation and function of macrophages and other phagocytes. These results also support the suggestion continuing anti-inflammatory state in DS and this process may explain the cause of recurrent infection in these patients.

It has been found that in children with DS <6 years of age, the levels of serum immunoglobulins did not differ from healthy controls (Nespoli *et al.*, 1993). In the present report, all the patients included the study was under 7 and their IgG, IgM and IgA levels were similar with healthy control group.

In conclusion, our findings demonstrate that the cell-mediated and humoral immune system parameters in children with DS were altered as compared to healthy children. We believe that these data will provide important contributions for future studies.

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