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Immune Thrombocytopenia in Children: What is the Role of Interleukin-4 and Tumor Necrosis Factor-Alpha?

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Abstract

***Background*: Immune thrombocytopenia (ITP) is a common acquired autoimmune disease in which autoantibody-coated platelets induce phagocytosis by macrophages. Complex immune dysregulation including Th1/Th2 imbalance is involved in the pathogenesis of this disease. Tumor necrosis factor-alpha (TNF-α) and interleukin 4 (IL-4) are cytokines produced by T cells and have various inflammatory, and immunomodulatory activities. *Aim:* To detect the changes in the level of TNF-α and IL-4in children with immune thrombocytopenia, and to correlate their levels with the disease course, severity, and response to treatment. *Subjects and Methods*: This is a case-control study that enrolled 100 subjects divided into 2 groups; patients' group: children with ITP and age- and gender-matched normal subjects as control. All participants were subjected to complete blood count, assessment of serum TNF-α, and IL-4 using ELISA technique. *Results:* There was a non-significant difference between patients and control groups (*p*-value 0.283) in serum IL-4; moreover, there was a non-significant difference in IL-4 levels among different severity grades of ITP. On the other hand, serum TNF-α was significantly higher in the patients' group than in the control group (*p*-value 0.002). Both TNF-α and IL-4 levels were significantly higher in newly diagnosed patients. *Conclusion*: TNF-α levels increase in patients with ITP. TNF- α and IL-4 are significantly higher in patients with newly diagnosed ITP.**

Keywords: Immune dysregulation; T Helper cells; ITP; TNF- α; IL-4

Introduction

Immune thrombocytopenia is an immune-mediated acquired disease, characterized by low circulating platelet count caused by the destruction of antibody-sensitized platelets in the reticuloendothelial system[(1)]. The pathophysiology of ITP is heterogeneous and complex. Research advances highlight that complex dysregulation of the immune system is involved in the pathogenesis of this condition[(2)]. This includes an increased number of T

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helper 1 (Th1) cells, decreased number or defective suppressive function of regulatory T cells[3], and platelet destruction by cytotoxic T lymphocytes[4]. The balance between cytokines leads to regulation of the immune system in normal states and is impaired in many autoimmune diseases. Many studies stressed on the role of serum cytokines in the pathogenesis of ITP and provide evidence to suggest that helper T lymphocytes polarize into Th1 and Th2 immune response[1]. The Th1/Th2 balance is important to normal human immunity. Th1 response is characterized primarily by the presence of cytokines IL-2, IFN-γ, and TNF-α, whereas the Th2 response produces IL-4, IL-5, IL-6, IL-10, and IL-13[5]. IL-4, one of the cytokines secreted by Th2, is a highly pleiotropic cytokine coded by a gene on chromosome 5 that is able to influence T-helper cell differentiation. It participates in the regulation of the immune system at multiple levels. IL-4 is also produced by, basophils, eosinophils, and mastocytes[6]. TNF-α, produced by Th1 cells, has a range of inflammatory and immunomodulatory activities[7]. It is secreted mainly by macrophages and T cells as a part of host defense against infection and is one of the cytokines that make up the acute phase reaction[8]. TNF gene is located on chromosome 6 in class III of the major histocompatibility complex (MHC)[9]. Polymorphisms of TNF promoters are associated with high levels of TNF and have been studied as determinants of susceptibility to numerous diseases[10]. Previous studies showed significant differences in serum cytokine levels between patients with ITP and healthy controls, indicating that cytokine disturbances might be involved in the pathogenesis of newly diagnosed ITP in both pediatric and adult patients[11]. In this study, we investigated two cytokines, TNF-α, produced by Th1 cells and IL-4 produced by Th2 cells among a cohort of Egyptian children with ITP in Suez Canal region and aimed to detect the correlation between their serum levels and both clinical presentation and laboratory data.

Subjects and Methods

All participants or their legal representatives provided written informed consent before participating in the study. The Ethics Committee of the Faculty of Medicine, Suez Canal University approved this study. Fifty pediatric patients (age range, 1 to 18 years), as well as 50 apparently healthy (age- and gender-matched) subjects were enrolled in the study and were recruited from the pediatric hematology clinic of Suez Canal University Hospital. All laboratory investigations were performed at the clinical Pathology Department, Suez Canal University Hospital.

Methods

All the participants in this study were subjected to complete history taking including disease duration, bleeding manifestations, complications, and family history. A thorough clinical examination was done with special emphasis on the site and shape of bleeding and the presence of organomegaly and lymphadenopathy. ITP is classified into: (1) newly diagnosed ITP: patients within 3 months of diagnosis. (2) Persistent ITP: disease duration between 3 and 12 months of diagnosis. (3) Chronic ITP: if the disease continues for more than 12 months[12]. Laboratory investigations were performed in-

cluding complete blood count (CBC) (Initially at diagnosis, at discharge, and after 3 months). A peripheral blood smear was examined. Measuring the serum level of tumor necrosis factor – α and IL-4 was done using ELISA technique. Venous samples were collected by standard venipuncture technique. Two ml of blood were collected on an EDTA tube for CBC using Sysmex XN-550.

Thrombocytopenia was divided according to severity to: Mild: platelets count is between 50 and 100 $\times 10^9$/l, moderate: platelets count is between 30 and 50×10^9/l, and severe: platelets count < 30×10^9/l[13]. Response to treatment was classified in the studied patients as: (a) Complete response (platelets $>100\times10^9$/l) at least 6 weeks post treatment. (b) Response (platelets between 30×10^9/l and 100) or double the baseline platelets count. (c) No response (platelets count < 30 $x10^9$/l or less than double base line platelets count[14]. Four mLs of blood were collected on plain tubes for TNF-α and IL-4 assay. Serum samples were stored at -20 °C until assay. Serum TNF-α (Cat. No. E0082Hu) and IL-4 (Cat. No. E0092Hu) ELISA kits were purchased from (Bioassay technology laboratory). The assays were performed according to the manufacturer's instructions.

Statistical Analysis

Data were analyzed using the statisti

cal package for social sciences (SPSS) for windows version 23.0 (SPSS, Chicago, IL, USA). Descriptive data were presented as mean ± SD or percentages. Fisher's exact test and chi-square test were used for statistical analysis of categorical variables. Because of skewed distributions, analysis of continuous variables was performed by non-parametric Mann-Whitney U-test and Kruskal Wallis test. Multivariate logistic regression analysis was applied to determine predictors for of ITP severity and chronicity.

Results

This case-control study had enrolled children with ITP attending the pediatric hematology clinic, Faculty of Medicine Suez Canal University hospital, Ismailia. Subjects in the control group were 21 females and 29 males; their ages ranged from 1.5 to 17 years old while patients with ITP were 25 males and an equal number of females with ages from 1 to 17 years old (Table 1).

Table1. Demographic characteristics of ITP patients and healthy controls

Variables	**Healthy Controls** (n= 50)	**ITP patients** (n= 50)	***p*-value**
Age (yrs.) mean ± SD median (range)	 9.02 ± 3.91 10 (1.5 – 17)	 6.66 ± 4.76 5 (1 – 17)	0.139 [a]
Gender, n (%) Male Female	 29 (58) 21 (42)	 25 (50) 25 (50)	0.42 [b]

[a] *P values are based on as Mann Whitney U test. Statistical significance at P <0.05*

[b] *P values are based on as chi-square test. Statistical significance at P <0.05*

Table 2 shows the clinical characteristics of the patients' group. About 44% of the patients had associated anemia and almost all of the patients had bleeding symptoms (92%); of those 43 % had Purpura, 24% had gum bleeding, 24 % had epistaxis, 4% had bleeding per rectum and finally 2% had menorrhagia (Table 2). The most administered medications were steroids (48%) and Eltrombopag (42%), other less commonly used drugs include IVIG (8%) and Azathioprine (2%). The mean treatment duration in patients with chronic ITP was 9.46±9.41 months, and 72 % of the patients showed a positive response to treatment (Table 3). There was a non-significant difference between ITP patients and healthy controls concerning their serum IL-4 (528.96±324.09 and 414.4±197.46 pg/ ml) respectively (p=0.283). On the other hand, ITP patients had significantly higher serum TNF-α levels (226.92±179.49 pg/ml) than healthy controls (181.22±160.87 pg/ml) (p= 0.002) (Table 4).

Table 2: Clinical characteristics of ITP pediatric patients

Variables	ITP patients (n=50)
Age at diagnosis (years)	
mean ± SD	4.49 ± 3.87
median (range)	3.4 (0.5 – 13)
Type of ITP: n (%)	
Acute	28 (56)
Chronic	22 (44)
Severity of ITP: n (%)	
Mild	4 (8)
Moderate	14 (28)
Severe	32 (64)
Platelet count (x10^9/l), (mean ± SD)	29.15 ± 26.92
History of anemia: n (%)	
Absent	28 (56)
Present	22 (44)
Severity of anemia (n=22): n (%)	
Mild	15 (68.2)
Moderate	7 (31.8)
Spleen status: n (%)	
Normal	45 (90)
Splenomegaly	3 (6)
Splenectomy	2 (4)
Bleeding symptoms: n (%)	
Absent	4 (8)
Present	46 (92)
Purpura	43 (86)
Bleeding per gum	12 (24)
Epistaxis	12 (24)
Bleeding per rectum	2 (4)
Menorrhagia	1 (2)

We found a statistically significant increase in the level of IL-4 in patients with newly diagnosed ITP (632.86 pg/ml ± 356.61) than in patients with

persistent and chronic forms of the disease (396.73±221.06) (*p*=0.02). While there was a non-significant difference in the levels of IL- 4 among different severity grades of ITP. In addition, there were non-significant relationships between levels of IL-4 and TNF-α and the presence of bleeding symptoms, presence of anemia, or response to treatment. Regarding TNF-α; its levels were significantly higher in patients with newly diagnosed ITP than in persistent and chronic ITP (287.68±191.13), (149.59±130.48) respectively; (p= 0.015). After applying post hoc test TNF-α level was significantly higher in patients with severe disease (271.13±179.53) followed by moderate (175.29±164.01) and then mild disease (54± 59.09) (*p*=0.013). (Table 5).

Table 3: Treatment characteristics of ITP in pediatric patients	
Variables	**ITP patients** (n=50)
Treatment regimen: n (%)	
Steroid treatment	24 (48)
Eltrombopag	21 (42)
IVIG	4 (8)
Azathioprine	1 (2)
Treatment duration in months	
mean ± SD	9.46 ± 9.41
median (range)	7.5 (0.5 – 36)
Response to treatment, n (%)	
Absent	14 (28)
Present	36 (72)

Table 4. Comparison of serum IL-4 and TNF-α between ITP pediatric patients and healthy controls			
Variables	**Healthy Controls** (n= 50)	**ITP patients** (n= 50)	***p*-value**
IL-4 (pg/ml),			
Mean ± SD	414.4 ± 197.46	528.96 ± 324.09	0.283 [a]
median (range)	327.5 (200 – 940)	422.5 (10 – 1105)	
TNF- α (pg/ml),			
Mean ± SD	181.22 ± 160.87	226.92 ± 179.49	**0.002** [a]
median (range)	99 (18 – 543)	177 (3 – 543)	

[a] *P values are based on as Mann Whitney U test. Statistically significance at P < 0.05*

Logistic regression analysis was done to determine the predictors of severe ITP and revealed that for every 10 pg/ml increase in the TNF- α level, the odds of having severe ITP increase by 4% (OR=1.004, *p*=0.025). Male sex was associated with an increase in the odds of having severe ITP 5.7 times compared to females (OR= 5.745, *p*=0.011) (Table 6). Regarding predictors of ITP chronicity, it was found that the odds of having chronic ITP increase by the decrease in the level of IL-4 and TNF-α serum levels. (OR

=0.997, *p*=0.015) (OR=0.995, *p*=0.01) (Table 7).

Discussion

Immune thrombocytopenia (ITP) is an autoimmune condition characterized by the presence of isolated thrombocytopenia. ITP is caused by IgG autoantibodies against platelet receptors[15]. In this study, we choose IL-4 and TNF- α to investigate their effects on ITP manifestations, severity, course, and response to treatment. In the present study patients with ITP

were 25 males and equal number of females with ages ranging from 1 to 17 years old. Although some reports showed that boys and girls are equally affected by childhood ITP[16,17]; other authors found a slight female predominance in ITP patients with a female: male ratio was 1.2/1 in a population-based registration of children with ITP that was performed in Norway[18]. On the other hand, Kuhne et al. found that the male: female ratio was in favor of males (54%) in children aged 2–5 years with female patients being older[19]. While 56% were newly diagnosed. Other reports estimated that the incidence of chronic ITP in childhood was 0.46 per 100,000 children per year with a prevalence of 4.6 per 100,000 children[18].

Table 5: Relationship between serum IL-4 and TNF-α and clinical characteristics of ITP pediatric patients

Variables	Serum IL-4 mean ± SD	*p*-value	Serum TNF- α mean ± SD	*p*-value
Type of ITP				
Acute	632.86 ± 356.61	0.024 [a]	287.68 ± 191.13	0.015 [a]
Chronic	396.73 ± 221.06		149.59 ± 130.48	
Severity of ITP				
Mild	337.5 ± 49.24		54 ± 59.09	
Moderate	430.21 ± 291.3	0.21 [b]	175.29 ± 164.01	**0.013** [b]
Severe	596.09 ± 341.21		271.13 ± 179.53	
Bleeding symptoms				
Absent	379 ± 58.94	0.69 [a]	84.75 ± 68.3	0.09 [a]
Present	542 ± 334.62		239.28 ± 181.16	
Anemia				
Absent	509 ± 319.11	0.58 [a]	195.86 ± 168.35	0.26 [a]
Present	554.36 ± 336.09		266.45 ± 189.25	
Response to treatment				
Absent	583.36 ± 313.76	0.43 [a]	286.71 ± 186.32	0.13 [a]
Present	507.81 ± 329.91		203.67 ± 173.86	

[a] *P values are based on as Mann Whitney U test. Statistical significance at P <0.05*

[b] *P values are based on as Kruskal Wallis test. Statistical significance at P <0.05*

Edslev et al. found that the platelet count is significantly decreased in both acute and chronic ITP, but it was maximally reduced in patients with acute ITP[20]. In our study, the mean platelet count was 29.15 ± 26.92 (x 10^9/l). Other studies reported lower platelet counts, Ĉulić et al., found that at the time of diagnosis, the platelet count was significantly low with (58%) of ITP

children having a platelet count below 10×10^9/l[11]. Other authors noted that The mean platelet count was 12.8 x 10^9/l in patients less than 15 years old, and 62% of ITP children had a platelet count of less than 10 x 10^9/l[21]. The previously mentioned low platelet count can explain the presence of anemia in 44% of our patients and obviously account for bleeding symptoms in (92%). The bleeding sites vary widely among different authors as we found some reports that 100% of patients had a petechial rash and 91·4% had epistaxis[22]. In the current study, there was a non-significant difference between ITP patients and healthy controls concerning their serum IL-4, which was in agreement with another study[23]. On the other hand, Talaat and colleagues stated that IL-4 level was significantly higher in ITP patients in comparison with the healthy controls. However, we found that there is a statistically significant increased level of IL-4 in patients with acute ITP than patients with persistent and chronic forms of the disease. Talaat and his collages reported similar results of positive correlation between IL-4 and acute ITP[22]. Some authors stated that IL-4 is a major role player in autoantibody production and focused on the important role of IL-4 for the differentiation of T-dependent B cells in addition to switching to different IgG isotypes. In addition, the increase in Th2 cytokine (IL-4 and IL-10) levels can affect the differentiation and survival of pathogenic B cells in ITP patients[23]. However, we could not find a significant difference in the levels of IL-4 among different severity grades of ITP. In addition, there was a non-significant relationship between levels of IL-4 and the presence of bleeding symptoms, presence of anemia, or response to treatment.

Table 6. Logistic regression analysis of determinants of severe ITP

Predictor	B	SE	OR (95% CI)	*p*-value
Constant	0.144	1.140		0.90
Age at diagnosis	-0.175	0.141	0.84 (0.637 – 1.108)	0.216
Gender *Male vs. female (R)*	1.748	0.690	5.745 (1.486 – 22.219)	**0.011** [a]
serum α-TNF	0.004	0.002	1.004 (1.001 – 1.008)	**0.025** [a]

*****CI; Confidence interval, [a] Statistical significance at p < 0.05*

Table 7: Logistic regression analysis of determinants of chronic ITP

Predictor	B	SE	OR (95% CI)	*p*-value
Constant	-0.244	0.953		0.798
Age at diagnosis	0.403	0.231	1.496 (0.952 – 2.352)	0.081
Gender *Male vs. female (R)*	-0.566	0.836	0.568 (0.11 – 2.923)	0.498
Serum IL-4	-0.003	0.001	0.997 (0.995 – 0.999)	**0.015** [a]
Serum α-TNF	-0.005	0.002	0.995 (0.991 – 0.99)	**0.01** [a]

***** CI; Confidence interval, [a] Statistical significance at P < 0.05*

Regarding TNF-α, ITP patients had significantly higher serum TNF- α levels than healthy controls, and its levels were significantly higher in patients

with acute than chronic ITP. This was in agreement with Talaat and co-authors who found that TNF-α has a positive correlation with acute ITP, and he also found that TNF-α levels are significantly elevated in patients with ITP compared to healthy controls[22]. These results are in line with 2 other studies that confirmed that increased levels of TNF-α in patients with ITP leads to macrophage activation, which is stimulated by autoantigens against platelets and results in T cell activation, then, activation of B cells. Moreover, The Th1/Th2 balance was found to be disturbed in patients with ITP, as the cytokines produced by Th1 (IFN-γ, TNF-α and IL-2) have a role in the cell-mediated inflammatory reaction, delayed hypersensitivity, and cytotoxic reaction activation, while Th2 cytokines (IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13) lead to excess antibody production[24,25]. The above-mentioned data can explain the results of logistic regression analysis that was done to detect the predictors of ITP severity and chronicity and revealed that the increase in the TNF-α level will increase the odds of diagnosing severe ITP; we also found that male sex was associated with increased risk of severe ITP. On the other hand, decreased level of both IL-4 and TNF-α was significantly associated with an increased risk of chronic ITP.

Conclusion

TNF-α level increases in patients with ITP. TNF-α and IL-4 are significantly higher in patients with newly diagnosed ITP. A high level of TNF-α can predict severe ITP at diagnosis, while a decrease in the level of both IL-4 and α-TNF can predict chronic ITP.

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