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Immune thrombocytopenia: serum cytokine levels in children and adults

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Background: Immune thrombocytopenia (ITP) is an immune-mediated platelet disorder in which autoantibody-coated platelets are removed from the blood by monocytic phagocytes and there is impaired platelet production. There is a delicate balance of specific cytokine levels, which has an important role in the immune system and is known to be deregulated in autoimmune diseases. This study was designed to investigate the differences in Th cytokine levels between children and adults with newly diagnosed ITP and to compare these profiles to those found in healthy, age-matched controls.

Material/Methods: The concentration of IL-1α, IL-2, IL-3, IL-4, IL-6, IL-10, TNF-α, IFN-α, and IFN-γ in serum specimens was analyzed by enzyme-linked immunosorbent assay.

Results: At the time of ITP diagnosis, children showed significantly lower serum levels of interleukin IL-2 and tumor necrosis factor TNF-α and higher serum level of IL-3 than healthy controls. Serum level of IL-4 in adult ITP patients was higher than those in control subjects. When compared with adults, children with ITP had lower serum level of IL-4, IL-6 and IFN-γ, and higher level of IFN-α.

Conclusions: Significant differences in serum cytokine levels between pediatric patients and healthy controls indicate that cytokine disturbances – especially changes in IL-2, IL-3 and TNF-α – might be involved in the pathogenesis of newly diagnosed ITP. TNF-α is the most informative variable for discrimination between healthy children and those with ITP.

Key words: immune thrombocytopenia • serum cytokines level • children • adults

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Background

Immune thrombocytopenia (ITP) is an immune-mediated platelet disorder in which autoantibody-coated platelets are removed from the blood by monocytic phagocytes, resulting in a remarkable decrease of platelet count, accompanied by impaired platelet production. Based on disease duration, ITP is classified into acute (newly diagnosed), persistent (3–12 months' duration) and chronic (>12 month duration) [1,2]. Both children and adults are prone to the development of persistent and chronic forms of the disorder, although the majority of childhood cases are acute and resolve successfully without therapy within 6 months [2,3]. Disease in adults is generally chronic (80%) and relapsing, and typically remains refractory to treatment [4].

The cytokine balance regulates the immune system under normal conditions and is impaired in many autoimmune diseases. Several studies support a role for serum cytokines in the pathogenesis of ITP, and provide evidence to suggest that helper T-lymphocytes polarize into Th1 and Th2 immune response [5,6]. Th1 response is characterized primarily by the presence of cytokines IL-2, INF-γ, and TNF-α, whereas Th2 response produces IL-4, IL-5, IL-6, IL-10 and IL-13. However, human Th1 and Th2 subsets are defined according to IFN-γ and IL-4 production because the synthesis of other cytokines is not stringently restricted to a single subset [5,6]. Recent studies of polymorphisms of genes that code for proteins included in proinflammatory immune response suggests that they contribute to the pathogenesis of ITP [7–10]. The ratio of Th1/Th2 to T1/T2 is significantly higher in ITP patients then in healthy controls [11,12]. Some studies have found evidence supporting Th2 polarization, suggesting that the Th1/Th2 ratio is directly correlated with platelet counts [13].

To further investigate the role and imbalance of Th cytokines in the pathogenesis of ITP, we measured serum concentration of IL-1α, IL-2, IL-3, IL-4, IL-6, IL-10, TNF-α, IFN-α, and IFN-γ in newly diagnosed pediatric and adult ITP patients.

Material and Methods

Patients

All participants or their legal representatives provided written informed consent before entry into the study, which was approved by the Ethics Committee of the Clinical Hospital Centre Split, Croatia, where the study was conduct. Forty-five pediatric patients (age range, 8 months to 14 years) and 19 adult patients (age range, 15–86 years), as well as 26 pediatric (age range, 1–12 years) and 24 adult healthy volunteers (age range, 15–50 years) were enrolled in the study. Patients and age-matched controls were Caucasians form south Croatia (Dalmatia). Heparinized venous blood samples were collected from each patient (at presentation time) and control subject. Serum samples were preserved at –80°C in aliquots for cytokine assays and were thawed only once.

Table 1. Distribution of platelet counts in children and adults with ITP at the time of diagnosis.

Platelet count	Children, N (%)*	Adults, N (%)*
No higher than 10×10^9/L	26 (58)	5 (26)
10.1–30.0×10^9/L	8 (18)	3 (16)
30.1–50.0×10^9/L	8 (18)	4 (21)
50.1–80.0×10^9/L	3 (6)	7 (37)
Total	45 (100)	19 (100)

* N – number of patients;% – percentage of patients (chi-square =10.622, df=3, P=0.0140). The normal range of platelet count: children – 170–420×10^9/L; adults – 150–400×10^9/L.

Inclusion criteria were: newly diagnosed ITP (no patients were in persistent or chronic phase of disease) with platelet count below 80×10^9/L, no comorbidity, and no obvious initiating and/or underlying cause of thrombocytopenia

Serum cytokines measurement

Serum levels of IL-1α, IL-2, IL-3, IL-4, IL-6, IL-10, TNF-α, IFN-α, and IFN-γ were determined using the quantitative sandwich enzyme-linked immunosorbent assay (ELISA) (Quantikine Immunoassay, R&D Systems Inc., Minneapolis, MN) [14].

Statistical analysis

Counted data are presented as numbers (N) and percentages (%), and difference between them was analyzed using the chi-squared test. Measured data are presented as medians and inter-quartile range (lower quartile to upper quartile), and were analyzed using the 2-group multivariate permutation test, which is the most appropriate test, considering group sizes and distributions. This test uses a 2-step procedure: (1) the overall P value is calculated, and (2) an individual permutation test for each variable is performed. However, the P values for the individual permutation tests are considered as significant only if overall P value is ≤0.05. Multivariate data analysis was done using the JRIP classification system in Weka software. The system generates rules that explain difference between groups under investigation. Data analysis was made using R and Weka software [15–17].

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Table 2. Serum cytokine levels in children with newly diagnosed ITP and in healthy controls.

Cytokine (pg/mL)	Healthy children*		Children with ITP*		P value**
IL-1α	2.21	(1.20 to 3.90)	3.40	(1.50 to 4.90)	0.2477
IL-2	29.10	(29.00 to 29.40)	28.50	(20.00 to 29.19)	0.0148***
IL-3	45.59	(42.10 to 47.21)	58.00	(45.97 to 64.00)	0.0405***
IL-4	8.55	(8.50 to 8.75)	8.15	(7.95 to 8.82)	0.1923
IL-6	4.44	(3.97 to 4.80)	4.24	(3.75 to 5.96)	0.9328
IL-10	17.27	(17.13 to 17.70)	17.20	(15.40 to 20.10)	0.5952
TNF-α	22.00	(21.05 to 23.95)	19.17	(17.00 to 22.81)	0.0025***
IFN-γ	46.13	(45.80 to 49.72)	45.00	(43.99 to 51.00)	0.0824
IFN-α	47.16	(12.50 to 70.19)	45.53	(39.05 to 68.48)	0.4736

* Data are presented as medians and inter-quartile range; ** P value of two-group permutation test; serum cytokine levels were compared using two-group multivariate permutation test, with the overall P value of 0.0095; *** statistically significant.

Table 3. Serum cytokine levels in adults with newly diagnosed ITP and in healthy controls.

Cytokine (pg/mL)	Healthy adults*		Adults with ITP*		P value**
IL-1α	2.60	(2.05 to 3.13)	3.10	(2.70 to 3.80)	0.0720
IL-2	26.00	(25.50 to 27.40)	27.50	(21.00 to 29.00)	0.2982
IL-3	64.25	(57.21 to 75.00)	60.00	(32.40 to 69.90)	0.2212
IL-4	9.65	(8.90 to 12.65)	11.50	(9.10 to 21.00)	0.0378
IL-6	7.64	(6.66 to 8.92)	8.00	(7.00 to 9.10)	0.3462
IL-10	17.65	(16.55 to 18.95)	16.60	(14.10 to 17.00)	0.0130
TNF-α	19.75	(18.50 to 21.33)	18.30	(16.60 to 21.00)	0.0914
IFN-γ	59.35	(43.06 to 62.40)	61.30	(43.97 to 68.30)	0.3718
IFN-α	20.40	(11.05 to 46.35)	23.45	(14.50 to 43.00)	0.7598

* Data are presented as means and inter-quartile range; ** P value of two-group permutation test; the data were compared using two-group multivariate permutation test, with the overall P value of 0.1530.

Results

Platelet count

At the time of diagnosis, the platelet count was significantly lower in children than in adults (p=0.014). Of the 45 children with ITP, 26 (58%) had a platelet count below 10×10^9/L, compared with 5/19 (26%) adult patients (Table 1).

Serum cytokine levels

Differences in serum cytokines level between healthy children and children with newly diagnosed primary ITP are presented in Table 2. Significantly lower levels of IL-2 (p=0.0148) and TNF-α (p=0.0025) and higher level of IL-3 were found in diseased children (p=0.0405). The level of other cytokines was not significantly different between these 2 groups of children (Table 2).

Based on the results of the 2-group multivariate permutation test, serum level of IL-4 in adult ITP patients was higher than in control subjects. No significant differences of other cytokine levels were observed (Table 3).

A comparison of serum cytokine levels at diagnosis in children and adults with ITP is presented in Table 4. Pediatric patients had significantly lower levels of IL-4 (p=0.0003), IL-6 (p=0.0006), and IFN-γ (p=0.0075), and significantly higher level of IFN-α (p=0.0095). There were no significant differences in the serum levels of other cytokines (IL-1α, IL-2, IL-3, IL-10, and TNF-α).

Multivariate analysis of cytokine levels in healthy children and children with ITP was done using the JRIP classification system in Weka software, which generates rules that explain differences between groups under investigation. The result of analysis with only 1 rule extracted suggests that the serum level

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Table 4. Serum cytokines in children and adults with newly diagnosed ITP.

Cytokine (pg/mL)	Children with ITP*		Adults with ITP*		P value**
IL-1α	3.40	(1.50 to 4.90)	3.10	(2.70 to 3.80)	0.7839
IL-2	28.50	(20.00 to 29.19)	27.50	(21.00 to 29.00)	0.5832
IL-3	58.00	(45.97 to 64.00)	60.00	(32.40 to 69.90)	0.3065
IL-4	8.15	(7.95 to 8.82)	11.50	(9.10 to 21.00)	0.0003
IL-6	4.24	(3.75 to 5.96)	8.00	(7.00 to 9.10)	0.0006
IL-10	17.20	(15.40 to 20.10)	16.60	(14.10 to 17.00)	0.1236
TNF-α	19.17	(17.00 to 22.81)	18.30	(16.60 to 21.00)	0.4046
IFN-γ	45.00	(43.99 to 51.00)	61.30	(43.97 to 68.30)	0.0074
IFN-α	45.53	(39.05 to 68.48)	23.45	(14.50 to 43.00)	0.0095

* Data are presented as means and inter-quartile range; ** P value of two-group permutation test; the data were compared using two-group multivariate permutation test, with the overall P value of 0.0283.

Table 5. JRIP rule and performace of classifier (confusion matrix) suggest that the serum level of TNF-α is the most informative variable for discrimination between healthy children and the children with ITP.

JRIP rule	Confusion matrix
IF (TNF-α ≥21 pg/mL) THAN Group = control children ELSE Group = children with ITP (42.0/4.0)	a b <-- classified as 22 4 | a = control children 10 38 | b = children with ITP

of TNF-α of 21.00 pg/mL is the most informative variable for discrimination between healthy children and children with ITP, with 81.08% of individuals correctly classified. The results of analysis are presented in Table 5 and in Figure 1.

Discussion

Immune thrombocytopenia is an autoimmune disease associated with cytokine deregulation. Results from the current study suggest differences in serum levels of some cytokines between newly diagnosed pediatric patients with ITP and healthy controls, and between pediatric and adult ITP patients, associated with differences in platelet count between pediatric and adult newly diagnosed ITP patients.

Our findings reveal a significantly lower platelet count in children than in adults with newly diagnosed ITP. Kühne et al., on behalf of the Intercontinental Cooperative ITP Study Group, studied the data of 1784 children and 340 adults and revealed that children and adults with newly diagnosed immune thrombocytopenia had similarities in presenting platelet counts [18]. Additionally, the levels of 3 different cytokines (decrease in IL-2 and TNF-α increase in IL-3) were different in children with ITP compared to healthy children. However, only 1 cytokine (increase in IL-4) was statistically different in adults with ITP

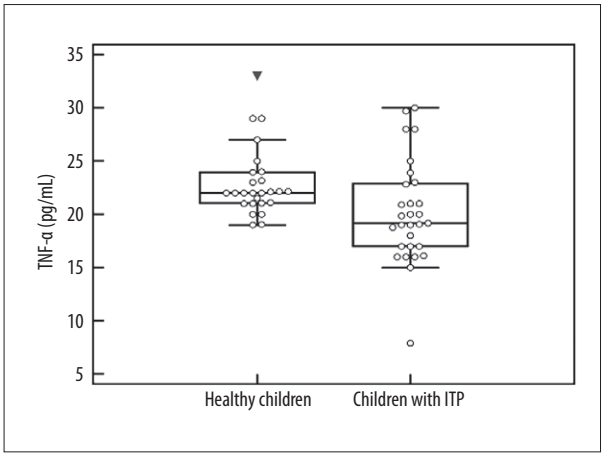

Figure 1. Serum levels of TNF-α (pg/mL) in healthy children and children with ITP.

compared to healthy controls. We did not find the differences in IL-10 serum concentrations between children with acute ITP and controls, in contrast to del Vechio, who found significantly higher levels of IL-10 in children with acute ITP. In the same study, serum levels of TNF-α and IL-2 did not differ significantly between patients and controls, but we found lower levels of TNF-α and IL-2 in our study [19]. Moreover, TNF-α was the most informative variable for discrimination between healthy children and children with ITP, with 81.08% of individuals

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correctly classified. Subsequently, both in our study and in the del Vechio et al. study, the relative levels of INF-γ and IL-6 did not differ significantly between patients and controls.

All of the above results suggest a Th2-like cytokine pattern in patients with ITP.

Additionally, our results show significantly higher levels of IL-4, IL-6, and IFN-γ in adults with ITP as compared to diseased children. This finding is in line with observation that aging may be accompanied by a gradual increase of certain cytokine levels, such as IFN-γ [20].

Conclusions

The finding of significant differences in serum cytokine levels between patients and healthy controls indicates that cytokine disturbances – especially changes in IL-4 – might be involved in the pathogenesis of newly diagnosed ITP in both pediatric and adult patients. Furthermore, TNF-α should be considered as a marker for discrimination between healthy children and those with ITP.

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