Clinical significance of serum cytokine levels and thrombopoietic markers in childhood idiopathic thrombocytopenic purpura

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Background. Biological markers useful for defining children with newly diagnosed immune thrombocytopenic purpura (ITP) who are likely to develop the chronic form of the disease are partially lacking. The purpose of this study was to assess the clinical role of both immunological and thrombopoietic markers in children with ITP and correlate their levels with different disease stages.

Materials and methods. We enrolled 28 children with ITP at the onset of their disease, who were followed-up for a whole year and divided according to whether their disease resolved within the 12 months (n=13) or became chronic (n=15), 11 subjects with chronic ITP off therapy for at least 1 month at the time of enrolment, and 30 healthy matched controls. Serum levels of T helper type 1 and 2 and T regulatory-associated cytokines, such as interferon γ, tumour necrosis factor α, interleukin (IL) 2, IL6, IL10, and thrombopoietin were measured in all children using quantitative immunoenzymatic assays, while reticulated platelets were evaluated by flow cytometric analysis.

Results. Serum IL10 levels were significantly higher in patients with an acute evolution of ITP than in either healthy controls ($p<0.001$) or patients with chronic progression of ITP ($p<0.05$). Reticulated platelet count and thrombopoietin levels were significantly higher in ITP patients at the onset of their disease, whether with acute resolution or chronic progression, than in healthy subjects ($p<0.01$; $p<0.001$), but did not differ between the groups of patients.

Conclusion. IL-10 seems to predict the clinical course of ITP, as it is significantly higher at the onset of disease in patients who obtain disease remission in less than 1 year.

Keywords: immune thrombocytopenic purpura, cytokines, thrombopoietin, reticulated platelets, children.

Introduction

Idiopathic (immune) thrombocytopenic purpura (ITP) is a heterogeneous clinical disorder characterised by immune-mediated platelet destruction. ITP is usually a benign, self-limiting disease in children[1]. However, approximately 20% of childhood newly diagnosed ITP progress to a chronic form defined according to standardised criteria[2].

The clinical differences between newly diagnosed and chronic ITP suggest the existence of different pathophysiological mechanisms in the two forms[1-3]. Many researchers have investigated the role of genetic factors[4,5], humoural and cellular immunity[1,6-8], and inadequate platelet production in the development of this condition[9], but failed to identify specific characteristics of children with ITP who will probably develop the chronic form of the disorder, mainly because of the study design and differences in patients' immunomodulating therapy.

Serum levels of T helper (Th) type 1, Th2 and T-regulatory associated cytokines, such as interferon (IFN) γ, tumour necrosis factor (TNF) α, and interleukin (IL) 2, IL6, IL10, and markers of thrombopoiesis, such as reticulated platelet count and thrombopoietin, were assessed in different phases of ITP in our patients and in healthy controls. We aimed to investigate whether these biomarkers might be considered predictors of ITP progression in children.

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Material and methods

Materials

Twenty-eight consecutive children who were referred to our Department for primary ITP were enrolled in this study at the onset of their disease before starting any treatment and were followed up for 1 year. Eleven children with chronic ITP (lasting at least 2 years) who were off therapy for at least 1 month, and 30 age- and sex-matched healthy controls were also recruited. The diagnosis of ITP was made following standardised guidelines[1,10-12].

The site and extent of bleeding symptoms at the onset of the disease were recorded and three different clinical phenotypes were identified according to national consensus[11]: (i) type I (asymptomatic-paucisymptomatic ITP), in the presence of clinical symptoms ranging from no bleeding to few petechiae and some bruises without mucosal haemorrhages; (ii) type II (intermediate ITP), in the presence of petechiae, bruising and mucosal haemorrhages; and (iii) type III (severe ITP), characterised by severe bleeding with organ impairment or life-threatening conditions (retinal or intracranial haemorrhage, or other severe internal haemorrhages, shock).

The parents of all children gave their consent to the study which was approved by the local Ethics Committee.

Cytokine assessment

A blood specimen was obtained from all children at the time of the first visit. Serum levels of IFNγ, TNFα, IL2, IL6, and IL10 were measured using a quantitative enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN, USA) following the manufacturer's instructions for sample collection, storage and assay procedure. The detection limits for the cytokines were 8 pg/mL for INFγ, 4.4 pg/mL for TNFα, 7 pg/mL for IL2, 0.7 pg/mL for IL6 and 3.9 pg/mL for IL10. Optical density values, obtained at two determinations, were converted into pg/mL by the Bio Rad ELISA data analysis software.

Reticulated platelets

Blood samples, collected into vacuum tubes containing EDTA as an anticoagulant, were centrifuged at 120 g for 10 minutes to obtain platelet-rich plasma. Platelets were fixed in 1% paraformaldehyde for at least 30 minutes at room temperature to minimise non-specific staining, washed twice and re-suspended at 50×10^9/L in phosphate-buffered saline containing 2 mM/L EDTA (pH 7.2). Fifty microlitres of this suspension were mixed with 10 μL of phycoerythrin-tagged monoclonal antibody against CD41 (Immunotech, Beckman Coulter, Marseille, France) and incubated at room temperature for 10 minutes in the dark. This suspension was then incubated with 1 mL of thiazole orange (Retic-COUNT, Becton Dickinson, San Jose, CA, USA) at room temperature in the dark for 1 hour. The samples were analysed on a flow cytometer (Epics XL-MCL Coulter Corporation).

Thrombopoietin

Plasma thrombopoietin concentrations were determined using an ELISA kit (Quantikine Human TPO Immunoassay, R&D Systems, Minneapolis, MN, USA) following the manufacturer's instruction for sample collection and assay procedure. The mean minimum detectable dose of this ELISA was 7.45 pg/mL. Optical density values were converted into pg/mL by the Bio Rad ELISA data analysis software.

Statistical analysis

The Stat View program (Abacus Concepts, Berkley, CA, USA) was used for statistical analysis. Data are expressed as medians and ranges. The Mann-Whitney U test (for unpaired data), the Kruskall-Wallis test (for subgroups analysis) and Spearman's rank correlation test were performed. p values ≥ 0.05 were considered statistically not significant (NS).

Results

The demographic and clinical characteristics of all enrolled children are summarised in Table I. Of the 28 patients who were enrolled at the onset of the disease, 13 had ITP lasting less than 12 months and formed group A, while the remainder had chronic progression of ITP and were defined as group B. Patients already affected by chronic ITP at the time of enrolment who had suspended treatment at least 1 month before entering the study were identified as group C. None of the patients had severe ITP; children in groups B and C showed a significantly greater prevalence of asymptomatic-paucisymptomatic forms of ITP compared to children in group A ($p<0.05$) (Table I).

There were no differences at study entry between

Table I - Patients' demographic and clinical characteristics.

Group*	N.	Male/ Female	Age (years)**	Asymptomatic-paucisymptomatic ITP Cases n (%)
A	13	8/5	3 (1-14)	7/13 (54%)
B	15	7/8	6 (1-14)	14/15 (93%)
C	11	6/5	6 (1-14)	10/11 (91%)
p		NS	NS	<0,05

Legend: ITP=Immune thrombocytopenic purpura; NS=Not significant. *A=patients with ITP lasting less than 12 months; B= patients with chronic course of ITP enrolled at the onset of their disease; C=patients with chronic ITP already present for at least 2 years at the time of enrolment. **Median (min-max)

patients with regards to platelet count, reticulated platelet count and thrombopoietin values (Table II). In contrast, the percentage of reticulated platelets and thrombopoietin levels were significantly higher in children in groups A and B than in healthy subjects ($p<0.01$; $p<0.001$). Levels of these thrombopoietic markers were not significantly different between patients in group C and individuals in the control group (Table II).

Among patients there was a significant inverse correlation between platelet count and TPO (r Rho=–0.39, $p<0.05$); in the other hand, reticulated platelet count correlated positively with the same biomarker (r Rho=0.48, $p<0.01$). No significant correlation was found between platelet count and reticulated platelets.

Data on serum cytokines levels in all children are summarized in Table III. Serum levels of INFγ, TNFα, IL2, and IL6 did not differ significantly between patients and controls. The concentration of IL10 was significantly higher in group A than in groups B and C and the control group ($p<0.05$, $p<0.01$ and $p<0.001$, respectively). The median values of IL10 were not significantly different between patients in group C and healthy subjects.

Discussion

In this study we recruited a series of newly diagnosed ITP cases at the onset of the disease, before starting any treatment, and a group of patients already affected by chronic ITP who received treatment (intravenous immunoglobulin or steroids) for their condition but that had suspended this treatment at least 1 month before enrolment into the study. In all these patients we sought for significant variations in immunological and thrombopoietic markers during the clinical progression (newly diagnosed or chronic) of their disease to determine the potential diagnostic and prognostic role of serum biomarkers in childhood ITP.

The demographic characteristics of our population

Table II - Platelets count, reticulated platelets (RPs) and thrombopoietin (TPO) levels in cases and controls.

Group	N.	Platelet count ($x10^9$/L)	RPs (%)	TPO (pg/L)
A	13	7.370 (1.000-40.000)	1 (0.5-8.0)	162 (43,5-278)
B	15	16 (3.860-32.900)	2,1 (0.1-8.0)	113 (57-268)
C	11	16 (4.360-57.700)	0,8 (0.1-8.5)	76 (52-411)
p		**NS**	**NS**	**NS**
Controls	15	252 (220-450)	0,4 (0,1 – 0,8)	62 (15-134)
p		**<0.001** Group A, B and C vs Controls	**<0.01** Group A vs Controls **<0.01** Group B vs Controls **NS** Group C vs Controls	**<0.001** Group A vs Controls **<0.001** Group B vs Controls **NS** Group C vs Controls

Legend: RPs=reticulated platelets; TPO=thrombopoietin; NS=not significant; A=patients with ITP lasting less than 12 months; B= patients with chronic course of ITP enrolled at the onset of their disease; C=patients with chronic ITP already present for at least 2 years at the time of enrolment.

Table III - Cytokines serum concentrations in cases and controls (median, range).

Group*	N.	IFNγ (pg/mL)	TNFα (pg/mL)	IL2 (pg/mL)	IL6 (pg/mL)	IL10 (pg/mL)
A	13	4.1 (1.5-11)	8.8 (3.7-22)	1.3 (0.5-3.5)	2.0 (1.3-2.8)	17 (1.7-18)
B	15	7.05 (1.7-32)	7.4 (2.8-23)	1.5 (0.5-4.0)	2.0 (0.5-9.6)	8.0 (2-17)
C	11	3.7 (0.3-10)	5.7 (1.3-8.2)	1.0 (0.5-3.2)	1.6 (0.1-21)	3.5 (0.6-11)
p		**NS**	**NS**	**NS**	**NS**	**< 0,05**
Controls	15	5 (3-7)	5 (3-9)	1 (0.5-3.2)	3 (2-4)	3 (1-7)
p		**NS** Group A, B and C vs Controls	**NS** Group A, B and C vs Controls	**NS** Group A, B and C vs Controls	**NS** Group A, B and C vs Controls	**< 0.01** Group A vs Controls **< 0.001** Group B vs Controls **NS** Group C vs Controls

Legend: * A=patients with ITP lasting less than 12 months; B= patients with chronic course of ITP enrolled at the onset of their disease; C=patients with chronic ITP already present for at least 2 years at the time of enrolment.

were similar to those reported by other authors[13-15]. In line with these other studies[15], our patients with chronic ITP showed a higher prevalence of asymptomatic-paucisymptomatic forms of ITP compared to children with newly diagnosed ITP.

We found that, at the onset of the disease, patients who went on to have acutely resolving ITP and those whose disease evolved into chronic ITP had slightly higher reticulated platelet counts and thrombopoietin levels than healthy children, but there were no differences within the studied group of patients. These parameters, which probably mirror ongoing massive platelet destruction, do not, therefore, seem to have a prognostic meaning with regards to ITP progression. Furthermore, when the same thrombopoietic markers were evaluated after disease onset in our group of children with chronic ITP (with the disease lasting at least 2 years), the levels in these patients were not different from those measured in controls. This is in contrast with the results of another paediatric study[16], in which it was demonstrated that after 6 months of ITP both reticulated platelet counts and thrombopoietin levels increased in children with disease progression. It was also shown that patients with ITP but a decreased rate of platelets had normal thrombopoietin levels[17]. These discrepancies can be explained by differences in case selection of children within the studies (i.e. disease duration, platelet rate).

In line with the results of another study[18], among all children affected by ITP we found a positive correlation between reticulated platelets and thrombopoietin and an inverse correlation between platelet count and thrombopoietin levels. We did not find a correlation between platelet count and reticulated platelets in our study which may be related to the fact that even if the number of circulating platelets is predominantly determined by platelet destruction, it can also be affected by platelet production. Moreover, the lack of standardisation of the technique for determining reticulated platelets might explain the inconsistency of our results with those reported in literature[19].

We observed that the concentrations of Th1 and Th2 cytokines were not significantly different within patients' groups and between cases and controls. However, IL10 serum concentrations measured in patients at the onset of their disease were higher than in healthy children and patients with ITP lasting for at least 2 years. Moreover, IL10 expression was significantly higher in the group of children with an acute course of the disease than in children who had chronically progressive ITP even if it was not possible to define a clear cut-off value of IL10 with prognostic relevance. Further prospective studies on larger population are needed to establish such a specific cut-off level and validate our results.

Some authors have previously described that serum levels of IL2, IFNγ and IL10 in children with

ITP were increased in some cases of chronic ITP, however in this study subjects were not grouped based on the clinical phases of the disease[7].

Studies on the expression of genes coding for Th1 and Th2 cytokines in childhood ITP showed that the majority of children with newly diagnosed ITP expressed *IL2* and *IFNG* (with or without *IL4*) cytokine genes *in vivo*[20], suggesting an early CD4 Th0 and Th1 cell activation and, more interestingly, underlining an important differentiating factor between the mild relapsing form and the aggressive chronic form of ITP in the presence or absence of IL10.

Moreover, high IL10-producing polymorphisms were found less frequently in patients with chronic ITP than in controls[4,21]. We are planning to investigate the association between genetic variants of *IL10* and levels of this cytokine in our same patients. Furthermore, it will be useful to carry out studies using cell separation methods to identify the source of IL10 in childhood ITP.

IL10 is an important immunoregolatory cytokine that is produced mainly by monocytes and lymphocytes[22]. It inhibits the formation of pro-inflammatory cytokines such as TNFα in T cells and monocytes[23], and down-regulates MHC class II expression in these latter cells[24]. In contrast to its inhibitory function on T cells and macrophages, IL10 stimulates the production of immunoglobulins and the expression of MHC class II antigens in B cells[25]. Moreover, IL10 is the main effector of IL10-secreting type I regulatory T cells in humans[26]. These cells are able to suppress antigen-specific effector T-cell responses via a cytokine-dependent mechanism and do, therefore, have a role in immunotolerance.

In conclusion, it is intriguing that high serum levels of IL10 were found at the onset of ITP in children with an acute disease course (and thus resolution), in agreement with the role of this cytokine in immunotolerance.

The serum level of IL10 in children with ITP at the onset of their disease may provide a promising indicator of the clinical progression of the disease.

The Authors declare no conflicts of interest.

References

1) Provan D, Stasi R, Newland AC, et al. International consensus report on the investigation and management of primary immune thrombocytopenia. Blood 2010; **115**: 168-86.
2) Rodeghiero F, Stasi R, Gernsheimer T, et al. Standardization of terminology, definitions and outcome criteria in immune thrombocytopenic purpura of adults and children: report from an international working group. Blood 2009; **113**: 2386-93.
3) Stasi R, Evangelista ML, Stipa E, et al. Idiopathic thrombocytopenic purpura: current concepts in pathophysiology and management. Thromb Haemost 2008; **99**: 4-13.
4) Wu KH, Peng CT, Li TC, et al. Interleukin 4, interleukin 6 and interleukin 10 polymorphisms in children with acute and chronic immune thrombocytopenic purpura. Br J Haematol 2005; **128**: 849-52.
5) Foster CB, Zhu S, Erichsen HC, et al. Polymorphisms in inflammatory cytokines and Fcgamma receptors in childhood chronic immune thrombocytopenic purpura: a pilot study. Br J Haematol 2001; **113**: 96-9.
6) McMillan R. The pathogenesis of chronic immune (idiopathic) thrombocytopenic purpura. Semin Hematol 2000; **37**: 5-9.
7) Semple JW, Milev Y, Cosgrave D, et al. Differences in serum cytokine levels in acute and chronic autoimmune thrombocytopenic purpura: relationship to platelet phenotype and antiplatelet T-cell reactivity. Blood 1996; **87**: 4245-54.
8) Wang T, Zhao H, Ren H, et al. Type 1 and type 2 T-cell profiles in idiopathic thrombocytopenic purpura. Haematologica 2005; **90**: 914-23.
9) Nugent D, McMillan R, Nichol JL, Slichter SJ. Pathogenesis of chronic immune thrombocytopenia: increased platelet destruction and/or decreased platelet production. Br J Haematol 2009; **146**: 585-96.
10) De Mattia D, Del Principe D, Del Vecchio GC, et al. Acute childhood idiopathic thrombocytopenic purpura: AIEOP consensus guidelines for diagnosis and treatment. Associazione Italiana di Ematologia e Oncologia Pediatrica. Haematologica 2000; **85**: 420-4.
11) De Mattia D, Del Vecchio GC, Russo G, et al. Management of chronic childhood immune thrombocytopenic purpura: AIEOP consensus guidelines. Acta Haematol 2010; **123**: 96-109.
12) Neunert C, Lim W, Crowther M, et al. The American Society of Hematology 2011 evidence-based practice guideline for immune thrombocytopenia. Blood 2011; **117**: 4190-207.
13) British Committee for Standards in Haematology General Haematology Task Force: Guidelines for investigation and management of ITP in adults, children, and in pregnancy. Br J Haematol 2003; **120**: 574-96.
14) Zeller B, Helgestad J, Hellebostad M, et al. Immune thrombocytopenic purpura in childhood in Norway: a prospective, population-based registration. Pediatr Hematol Oncol 2000; **17**: 551-8.
15) Zeller B, Rajantie J, Hedlund-Treutiger I, et al. Childhood idiopathic thrombocytopenic purpura in

the Nordic countries: epidemiology and predictors of chronic disease. Acta Paediatr 2005; **94**: 178-84.

16) Rajantie J, Javela K, Joutsi-Korhonen L, et al. Chronic thrombocytopenia of childhood: use of non-invasive methods in clinical evaluation. Eur J Haematol 2004; **72**: 268-72.
17) Houwerzijl EJ, Louwes H, Esselink MT, et al. Increased glycocalicin index and normal thrombopoietin levels in patients with idiopathic thrombocytopenic purpura with a decreased rate of platelet production. Haematologica 2005; **90**: 710-1.
18) Kaushansky K. The molecular mechanisms that control thrombopoiesis. J Clin Invest 2005; **115**: 3339-47.
19) Pons I, Monteagudo M, Lucchetti G, et al. Correlation between immature platelet fraction and reticulated platelets. Usefulness in the etiology diagnosis of thrombocytopenia. Eur J Haematol 2010; **85**: 158-63.
20) Mouzaki A, Theodoropoulou M, Gianakopoulos I, et al. Expression patterns of Th1 and Th2 cytokine genes in childhood idiopathic thrombocytopenic purpura (ITP) at presentation and their modulation by intravenous immunoglobulin G (IVIg) treatment: their role in prognosis. Blood 2002; **100**: 1774-9.
21) Saitoh T, Kasamatsu T, Inoue M, et al. Interleukin-10 gene polymorphism reflects the severity of chronic immune thrombocytopenia in Japanese patients. Int J Lab Hematol 2011; **33**: 526-32.
22) Mosmann TR. Properties and functions of interleukin-10. Adv Immunol 1994; **56**: 1-26.
23) Fiorentino DF, Zlotnik A, Mosmann TR, et al. IL-10 inhibits cytokine production by activated macrophages. J Immunol 1991; **147**: 3815-22.
24) Wanidworanun C, Strober W. Predominant role of tumor necrosis factor-alpha in human monocyte IL-10 synthesis. J Immunol 1993; **151**: 6853-61.
25) Rousset F, Garcia E, Defrance T, et al. Interleukin 10 is a potent growth and differentiation factor for activated human B lymphocytes. Proc Natl Acad Sci USA 1992; **89**: 1890-3.
26) Roncarolo MG, Gregori S, Battaglia M, et al. Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. Immunol Rev 2006; **212**: 28-50.

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