Cytokine Profile of Type 1 and Type 2 T Helper Cells in Children with Acute Immune Thrombocytopenia

Bhaskar Vanita*, Nangia Anita, Sharma Sunita, Chandra Jagdish, Seth Anju
Department of Pathology and Paediatrics, Lady Hardinge Medical College and Associated Hospital, New Delhi, India

ABSTRACT

BACKGROUND
Immune thrombocytopenia is an autoimmune disorder characterized by low circulating platelet count caused by destruction of antibody sensitized platelets in reticuloendothelial system. It can be primary or in association with other disorders. It was recently reported that dysfunctional cellular immunity may play an important role in pathophysiology of ITP.

AIM
To study pretreatment and posttreatment cytokine profile of Th1 (IL - 2, IFNγ) and Th2 (IL-4, IL-10) T cells in children with acute immune thrombocytopenia.

SETTINGS AND DESIGN
Interventional and observational study.

MATERIALS AND METHODS
A total of 30 patients were evaluated in the study. The tests performed included are complete haemogram including platelet count, peripheral smear examination (P/S), Bone Marrow Aspirate examination (BMA), Serum Anti-platelet Antibody (SAPA) and serum cytokine levels (Th1=IFNγ, IL2 and Th2=IL4, IL10) using ELISA method. To study the sequential changes in serum cytokine levels pre and post treatment with immunomodulatory treatment, CBC including platelet count and serum cytokine levels were analysed on the day 0 *i.e* pre-treatment and then on day 1, day 4 and day 30 post-treatment. PS, BMA and SAPA were performed only once *i.e* on day 0.

STATISTICAL ANALYSIS
The results were analysed using paired t-test. The "t" values obtained were used to get the probability (p values) values. "t" Values for various tests were analysed to record the levels of significance. Statistical software used was SPSS version 15.0

RESULTS
Level of Th1 cytokines *i.e* IFNγ and IL-2 were found to be high and Th2 cytokines *i.e* IL-4 and IL-10 were low in all the patients with active disease who have not received treatment. After immunomodulatory treatment, Th1 level got decreased and Th2 levels increased significantly on day 1 after treatment which continued on day 4, 7, 30.

CONCLUSION
The present study found an increase in Th1 cytokine-IFNγ and IL2 and decrease in Th2 cytokine–IL4 and IL10 levels in patients with active disease which get normalised by immunomodulatory treatment.

KEYWORDS
Acute immune thrombocytopenia, Cytokines, Immunomodulatory, ELISA, Interleukin (IL), Interferon gamma (IFNγ), Reticuloendothelial system

*Corresponding Author:
Vanita B, Department of Pathology and Paediatrics, Lady Hardinge Medical College and Associated Hospital, New Delhi, India;
E-mail: adilevanita@gmail.com

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INTRODUCTION

Immune Thrombocytopenia (ITP) is an autoimmune disorder characterized by low circulating platelet count caused by destruction of antibody sensitized platelets in reticuloendothelial system.[1-3] International Working Group (IWG) defines primary ITP as thrombocytopenia in absence of underlying cause or disorder and value of platelet count less than 100 X 10^9/L. The disease may also be classified according to patient's age–adult type and childhood type, and depending on duration of thrombocytopenia into acute, persistent and chronic. The incidence of ITP in children is about 40-80 cases per 1,000,000 per year.[4] It may occur in isolation (primary) or in association with other disorders (secondary), such as autoimmune diseases including antiphospholipid antibody syndrome, viral infections such as HCV, HIV, collagen vascular diseases and certain drugs.[5] The immunologic platelets destruction has long been believed to be the basic defect in ITP. It occurs as a result of autoantibody or immune complex deposition on platelet membrane. Platelets are sensitized by these auto-antibodies, predominantly Immunoglobulin (IgG) but some times of IgM class. Antibody coated platelets then binds to macrophage Fc receptor in spleen and to some extent in liver and are destroyed.[6] Most commonly implicated antigen is Glycoprotein (GP) IIb/IIIa and less commonly others, such as gp Ib/V/IX. Recently, it has been postulated that dysfunctional cellular immunity plays an important role in pathophysiology of ITP.[7] These includes the presence of activated platelet specific auto reactive T-cell that recognizes and responds to autologous platelet antigen and so drive the generation of platelet reactive autoantibody by B-cells. In addition, presence of T-cell mediated cytotoxicity and complement mediated lysis of platelet is also seen in ITP.[8-10] The different T-cell subsets are recognized by the type of cytokine secreted by them like Th1 and Th2. Acute to chronic ITP forms a single continuous disease spectrum. Approximately 7% to 28% of children with acute ITP develop chronic ITP.[11] However, there are no specific parameters or tests which can predict the conversion or progression to chronic ITP. Few studies have shown *in vitro* cellular immune defect in patients of both acute and chronic ITP but little is known regarding serum cytokines in acute ITP, especially in children and their role in pathogenesis of ITP. Cytokine levels may assess the patients more likely to progress to chronic ITP and their response to immunomodulatory therapy.[12-15]

MATERIALS AND METHODS

The study was conducted in department of pathology and department of paediatrics, lady Harding medical college and associated Kalawati Saran Children's Hospital (KSCH), New Delhi from November 2010 to February 2012. A total of 30 newly diagnosed ITP patients of paediatric age groups who fulfilled the following inclusion criteria were included in the study after taking informed consent from their parents or guardian.

Inclusion Criteria

Children between 6 months–18 years of age presenting with bleeding manifestations suggestive of acute ITP for duration 0–3 months (any one or more): Generalized petechiae, Purpura, Gum bleeding, Bleeding from mucous membrane/s. Patients with platelet count <1 lakh/µl. Patients with megakaryocytic hyperplasia on bone marrow aspiration.

Exclusion Criteria

Patients who refuse consent, known case of acute ITP patients, who are on treatment, patients with recent history of blood and platelet transfusion, patients with history/laboratory investigations suggestive of secondary ITP, patients with congenital thrombocytopenia.

The cases were subjected to complete haemogram with platelet count (fully automated haematology analyser sysmex KX21), peripheral smear and Bone Marrow Aspirate (BMA) (Wright stained), serum antiplatelet antibody levels (ELISA method), serum cytokine level of IL-2, IFN y–Th 1 subset and IL-4, IL-10-Th2 subset (ELISA method). Complete haemogram with platelet count and the cytokine levels were performed in all patients on day 0 and day 1,4 and 30 after immunomodulatory treatment. The patients were treated as per the standard protocol/guidelines and hospital policy.

RESULTS

The age of the patients ranged from 7 months to 18 years, with mean of 7.38 years and standard deviation of 4.83. Maximum patients were in the age group of 4-8 years. Out of 30 patients studied 17 (56.66%) were male child with male to female ratio of 1.3:1. Almost all the patients 28/30 (93.33%) presented with generalised petechiae associated with one or more other symptoms at the time of admission and out of these, 6 (20%) patients had only generalised petechiae. 1 (3.33%) patient presented exclusively with mucosal bleed and malena each. None of the patient had intracranial bleed. History of preceding viral illness was present in 5/30 (16.66%) patients. On examination none of the patients had lymphadenopathy, splenomegaly and/or hepatomegaly. All patients had a normocellular to mildly hypercellular marrow. Erythroid series showed a normoblastic reaction in almost all the patients 29 (96.66%) accompanied by hyperplasia only in one patient. Myeloid series showed normal maturation with only 5 (16.66%) patients having increased eosinophilic precursors. Megakaryocytic hyperplasia, with presence of both immature and mature forms was observed in all the patients 30/30 (100%). Platelet budding was present in 26 patients (86.66%). Morphological abnormalities in form of hypolobated nuclei in mature forms were found in 4 patients (13.33%) and cytoplasmic vacuolization was present in 2 patients (6.66%). The serum antiplatelet antibodies against Glycoprotein (Gp) IIb/IIIa, Gp Ib/IX, and GpIa/IIb were studied only on day 0.8/30 patients (26.66%) had circulating antibodies only against Gp IIb/IIIa. Platelet counts on day 0 were low in all the patients with a range of 1000–31000/µl. The mean platelet count was 9733 (± 7528.95)/µl. Post treatment levels of mean platelet count showed increasing trend from day 0 to day 1, day 4 and day 30. The increment in counts were found to be statistically significant with p-value <0.001 on all days.[16-19]

Th1 Cytokines

The mean values of IFNγ levels were found to be 75.03 (± 49.47) pg/ml in patients on day 0 (pre-treatment). The mean levels showed a marked decrease following treatment on all the follow up days *i.e* on day 1,4 and 30 which were found be statistically significant (p<0.001). The mean levels of IL-2 were high on day 0 *i.e* 7.89 (± 6.75) pg/ml. The values showed a decreasing trend which was statistically significant on all the follow up days *i.e* day 1,4 and 30 (p=0.01, 0.004, 0.02) respectively. There was slight increase in values on day

30, however the values were lower than pre-treatment levels.

Th2 Cytokines

The mean levels of IL-4 on day 0 were found to be 2.5 (± 0.63) pg/ml. Post-treatment the values showed an increasing trends which was found to be statistically significant ($p<0.001$) on all days 1,4 and 30. The mean levels of IL-10 were found to be 3.35 (± 0.63) pg/ml. The day 1, 4 and 30 values post treatment showed an increasing trend which was found to be statistically significant ($p=0.01$, <0.001, <0.001 respectively). Although all cytokines showed consistent increasing (Th2 cytokine–IL-4, IL-10) or decreasing trends (Th1-IFNγ, IL-2) post treatment yet maximal response was seen on different days (Figure 1). The cytokine ratios were also studied.

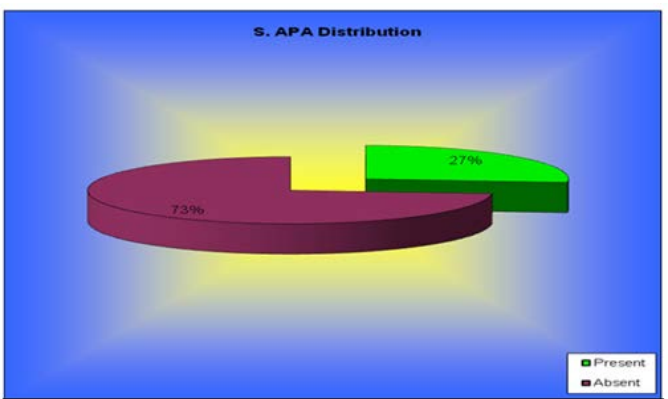

Figure 1. Anti-platelet Antibodies in (8/30) 27% of patients.

Parameters studied sequentially on day 0, day 1, day 4 and day 30

Platelet counts (/µl) (Table 1).

Days	Range (/µl)	Mean platelet count (± SD) (in/µl)	P-values
Day 0	1000 -1000	9733 (± 7528.95)	-
Day 1	4000-55000	17733 (± 13625.37)	<0.001
Day 4	5000-54000	24467 (± 12886.06)	<0.001
Day 30	8000-1,20,000	53333 (± 32241.90)	<0.001

Table 1. Showing Distribution of Platelet Counts.

Serum cytokine levels

Results of IFNγ (in pg /ml) tabulated in (Table 2 and Figure 2).

Days	Range	Mean (± SD)	P-value
Day 0	25-207	75.03 (± 49.47)	-
Day 1	10-180	46.70 (± 40.50)	<0.001
Day 4	10-177	47.13 (± 42.06)	<0.001
Day 30	10-110	33.41 (± 27.37)	<0.001

Table 2. Distribution of IFNγin Pg/ml.

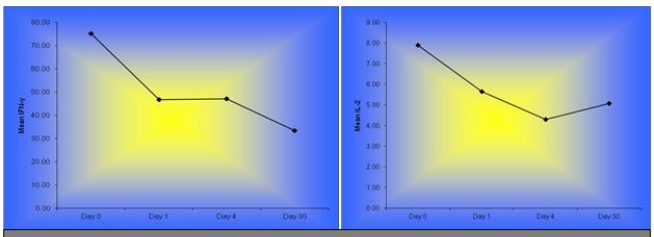

Figure 2. Showing Trend of IFNγ and IL-2 Pre and Post Treatment.

Results of IL-2 in Pg/ml (Table 3).

Days	Ranges	Mean (± SD)	P - value
Day 0	0-20	7.89 (± 6.75)	-
Day 1	0-20	5.65 (± 5.29)	0.01
Day 4	0-14	4.29 (± 5.21)	0.004
Day 30	0-12.5	5.08 (± 4.76)	0.02

Table 3. Distribution of IL-2 in pg/ml

Results of IL-4 (in Pg/ml) (Table 4 and Figure 3).

Days	Ranges	Mean (± SD)	P - value
Day 0	1.5-3.8	2.5 (± 0.63)	-
Day 1	1.6-5.4	3.11 (± 0.95)	0.001
Day 4	2-5.8	3.9 (± 1.27)	0.001
Day 30	1.8-8.9	5.9 (± 2.22)	<0.001

Table 4. Distribution of IL-4.

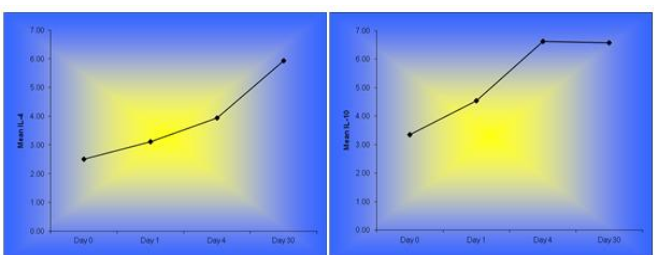

Figure 3. Showing Trends of IL-4 and IL-10 Pre and Post Treatment.

Result of IL-10 (in Pg/ml) (Table 5).

Days	Ranges	Mean (± SD)	P - value
Day 0	0-8	3.35 (± 3.28)	-
Day 1	0-10	4.54 (± 2.95)	0.01
Day 4	05-10	6.61 (± 1.28)	<0.001
Day 30	05-10	6.57 (± 1.19)	<0.001

Table 5. Distribution of IL-10.

DISCUSSION

Chong BH, et al., found in his study that acute ITP is equally distributed between male and female. Douglas B, et al., found that the affected children with peak age of 5 years

presenting with sudden onset of petechial or purpura a few days or weeks after an infectious illness and also found that boys and girls were equally affected. Nugent DJ, et al., studied on childhood ITP and found that the peak age group was 2–5 years, a period when children experience the greatest frequency of viral infections with equal incidence in males as in females. However, Montgomery R, et al., found that recent history of viral illness was much higher and present in 50%-60% of cases of childhood ITP. Our study showed slight male predominance with presence of preceding illness in only 16.66 % patients.

Chandra J, et al., studied bleeding manifestations in 58 children of immune thrombocytopenia with platelet count <20,000/µl. They found that patients with platelet counts >10,000/µl presented with either no or mild cutaneous bleeds. The patients with count <10,000/µl presented with more frequent bleeding episodes in form of cutaneous bleeds associated with bleeding at other sites like mucosal bleeding. However, the present study found that there is not much difference in presentations of patients with platelet counts >/<10,000 / µl.

Increased number of megakaryocytes were observed in studies performed by George, et al., and Levine, et al., studied alteration of megakaryocytes in thrombocytopenia in bone marrow aspirate of 19 ITP patients. Megakaryocytic hyperplasia with presence of mature and immature forms was seen in 18/19 (94.73 %) patients and platelet budding was seen in 12/19 (63.15 %) patients. Findings of present study are similar to their study. However, they observed cytoplasmic vacuolization in 4/19 (21.05 %) and hypolobation in only 1/19 (5.26%) patients, unlike our study.

Tests for serum antiplatelet antibody against Glycoprotein (Gp) IIb/IIIa, Gp Ib/IX, Gp Ia/IIb were performed in all the patients at day 0 (pre-treatment) and found to be present in 8/30 (26.66%) of the patients. The antibodies were directed against one platelet antigen *i.e* GpIIb/IIIa, only. Present study showed results similar to study conducted by Berchtold P, et al.[19] They studied the auto antibodies against platelet membrane Glycoprotein (Gp) in 15 children with acute and 24 children with chronic ITP. They detected platelet auto antibodies (anti–GpIIb/IIIa) in 4/15 (26.7%) in acute ITP patients and in 14/24 (58.3%) in chronic ITP patients. None of the patients with either chronic or acute ITP had detectable auto antibodies to GpIb / IX.

Malinowska, et al., studied 18 children of Chronic ITP and found a significant increase in platelet counts post treatment (anti-D infusion), 20 hours post infusion in 10/18 children, 96 hrs in three children and 168 hrs in one child and found that the mean duration of response was four weeks. In our study, however the response in most patient was seen only by day 4 (96 hours) though maximal response was similar at 4 weeks.

Semple JW, et al., studied difference in serum cytokine levels of IFNγ, IL-2, IL-4 and IL-6 in 11 children with acute and 23 children with chronic ITP. They found that Th1 cytokine marker IFNγ and IL-2 were increased in all patients of ITP, the levels being more in chronic ITP patients as compared to acute ITP patients. However, the serum levels of IL-4 and IL-6 were undetectable. Anderson J, et al., studied that the children with ITP had a Th1 type of cytokine pattern with elevated levels of IFNγ, IL-2 and TNF-β and low IL-4 and IL-6. The authors observed that ITP is associated with a Th1 type of T helper cytokine response while cytokines of type Th2 are down regulated. Ogawara H, et al., studied 42 patients of chronic ITP by flow cytometry to assess intracellular IFNγ and IL-4 levels and found that the mean level of intracellular IFNγ in Th1 cells was higher in patients with active disease than those of controls. Guo C, et al., investigated the correction of Th1 dominant cytokine profiles by high dose Dexamethasone in 52 patients with chronic ITP. The pre-treatment plasma levels of both IFNγ and IL-2 were significantly increased. After high dose Dexamethasone treatment, IFNγ and IL-2 were decreased significantly and attained the levels comparable to controls but their levels reduced again in the relapsed patients. This is similar to our study.

Semple JW, et al., found that IL4 serum levels were undetectable in all the children with acute and chronic ITP. Anderson J, et al., found that IL-4 levels were significantly decreased in patients with ITP. Mouzaki, et al. 2002 found that IL-10 gene expression was negatively correlated with disease activity. It was found to be highly expressed in acute phase and during relapse and expression decreased after IVIg infusion and reached zero level at follow up. Wang, et al., 2005 studied the type 1 and type 2 T cell profile in 30 adult patients of chronic idiopathic thrombocytopenic purpura and suggested that active ITP patients had significantly higher Th1/Th2 ratio because of a decrease in the number of Th2 cells and also concluded that Th1/Th2 ratio approached normal range when the disease was in remission. Guo C, et al., investigated the correction of Th1 dominant cytokine profiles by high dose Dexamethasone in 52 patients with chronic ITP. Along with IFNγ and IL-2, they also studied IL-4 and IL-10 levels in patient's plasma, which were significantly decreased in the patients as compared to controls. After high dose Dexamethasone treatment, IL-4 and IL-10 increased significantly and attained the levels comparable to controls but levels reduced again in the relapsed patients. Chang et al., measured the plasma levels of IL4 and IL-10 in 35 chronic ITP patients. He found that plasma level of IL-4 was significantly decreased in patients with active disease as compared to patients in remission and controls. The levels of IL-10 were significantly decreased in patients with active disease and in patients with non-remission as compared to patients on remission and controls. Present study also concluded the same.

The present study found altered cytokine levels in all patients of acute ITP at the time of diagnosis. However, antiplatelet antibodies were present in only 8/30 (26.66%) of the patients. Thus, showing the dysregulation in cytokine secretion as an event which occurs earlier to manifestation of Humoral immune response. Following immunomodulatory treatment the levels of Th1 cytokines goes down and Th2 cytokines increases. This heralds an improvement in clinical status and platelet count. The single time post cytokine levels (IFNγ and IL-4) done on day 1 are capable of predicting response to treatment and sequential evaluation is not needed.[20]

CONCLUSION

The present study found an increase in Th1 cytokines, IFNγ and IL-2 and decrease in Th2 cytokines IL-4 and IL-10, in all paediatric patients of acute ITP at time of diagnosis. This emphasizes the T-cell dysregulation as the early event in pathophysiology of acute ITP with appearance of platelet antibodies as a late phenomenon. The decrease in Th1 cytokines and increase in Th2 cytokine post treatment is an excellent indicator of response to treatment. This trend is observed on all days, *i.e* day 1,4 and 30 hence, sequential determination of values is not needed. The IFNγ and IL-4 are better predictors of response to immunomodulation as compared to IL-2 and IL-10. However, the maximal response in values of IFNγ and IL-4 are achieved on different days. Hence, their assessment performed pre-treatment and 24

hours *i.e* Day 1 post treatment is a better and accurate predictor of response to immunomodulatory treatment, obviating the need for sequential determination. However, further studies with larger groups of patients are needed to determine the significant cut offs for diagnostic and prognostic use of cytokines in patients with acute ITP. These values may also be used in planning for cytokines associated immunomodulatory therapy in these patients.

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