Level of IL-16 and Reticulated Platelets Percentage during the Clinical Course of Immune Thrombocytopenic Purpura in Children

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Immune thrombocytopenic purpura (ITP) is an immune-mediated acquired disease with transient or persistent decrease of thrombocytes number in the blood. Cytokines play important roles in the immune regulation and are known to be deregulated in autoimmune diseases. This study aimed to investigate serum IL-16 levels in relation to reticulated platelets in children with ITP and platelet count. Twenty six children with ITP (11 with newly diagnosed ITP, 9 with persistent ITP and 6 with chronic ITP) and 12 age-matched healthy children controls were studied. Serum level of IL-16 and reticulated platelets count were assessed by Enzyme Linked Immunosorbent Assay (ELISA) and flow cytometry respectively. Serum IL-16 levels were significantly higher in patients as compared to controls (P<0.001). Within patients, the levels were higher in newly diagnosed compared to persistent and chronic ITP (P<0.01) and (P<0.001) respectively. IL-16 levels were also significantly higher in persistent ITP compared to chronic ITP (P<0.001). Reticulated platelets were also elevated in patients compared to controls and the increase was significant in newly diagnosed group (P<0.05). Negative correlation was found between IL-16 level and reticulated platelets and platelets counts (r=-0.248, P=0.028, r=0.274 P=0.25) respectively. It is concluded that IL-16 may be valuable in predicting the clinical course of pediatrics ITP. Measurement of reticulated platelets may provide significant information about thrombopoietic activity during the clinical course of ITP in children.

Interleukin 16 (IL-16), formerly known as lymphocyte chemo attractant factor or LCF, is a proinflammatory cytokine that is a major chemotactic signal for CD4+ T lymphocytes, monocytes and eosinophils. It plays a role in trafficking of several immune cells (Conti, 2002). IL-16 was originally identified as a homotetramer consisting of individual 14 kDa monomers of 130 amino acids each (Cruikshank et al., 1994). Sources of IL-16 include epithelial cells, mast cells, lymphocytes, macrophages, synovial fibroblasts, and eosinophils (Laberge et al., 1999). The gene for human IL-16 maps to chromosome 15 and the sequence displays > 90% homology to those of various nonhuman primates (Kim, 1999).
It is synthesized as a precursor molecule that is cleaved by caspase-3 to produce a pro-IL-16 molecule that functions as a regulator of T cell growth, and a secreted peptide that functions as a CD4 and/or CD9 ligand for induction of cell motility and activation. IL-16 has been predominantly studied as a contributing factor in the orchestration of an immune response (Richmond et al., 2014).

Many of studies have implicated IL-16 in exacerbation of infectious, immune-mediated, and autoimmune inflammatory disorders, including asthma (Gantner et al., 2002), atopic dermatitis (Lee et al., 2012), rheumatoid arthritis (Franz et al., 1998) systemic lupus erythematosus, (Xue et al., 2009), multiple sclerosis (Biddison et al., 1997) and viral infections (Glass, 2006). However, little is known about how the IL-16 levels in the ITP change during the course of the disease.

Platelet membrane has a large number of glycoproteins that are essential for their normal functioning. Some glycoproteins are present in the resting state as well as after stimulation e.g. CD41. The CD41 antigen is expressed on platelets and megakaryocytes. The expression of CD41 is associated with early stages of hematopoiesis and is highly regulated during hematopoietic development (Mitjavila et al., 2002).

Reticulocytes are the youngest circulating platelet population and they contain abundant amounts of mRNA. It has been shown that the abundance of reticulated platelets mirrors the rate of megakaryopoiesis in the bone marrow, and conditions with high platelets turnover can be discriminated from bone marrow insufficiency by a significantly increased percentage of reticulated platelets in the peripheral blood (Richards & Baglin, 1995).

This study evaluates serum level of IL-16 and reticulated platelets in children with ITP to determine whether these parameters differ in newly diagnosed, persistent and chronic ITP, or correlate with platelet count and assess their significance as markers in different stages of ITP.

Subjects and Methods

Subjects

This study was conducted on 26 children with ITP (14 females and 12 males) attending Pediatric Department, Benha University Hospital, during the period from June 2014 to October 2014. Their age ranged from 7 months to 12 years. 12 healthy children (Their age ranged from, 9 months to 13 years) were tested as control group. The patients were classified into three groups.

Newly diagnosed ITP group: Consist of 11 children with ITP within the last 3 months.

Persistent ITP group: Consist of 9 children with ITP and their cases lasting between 3 to 12 months from diagnosis.

Chronic ITP group: Consist of 6 children who were defined as persisting thrombocytopenia of less than 100x10^9/L lasting for more than 12 months (Rodeghiero, 2009).

History of preceding infection and vaccination, platelet count, bleeding manifestations and pervious treatment were recorded.

The study was approved by Research Ethics Committee in Benha Faculty of Medicine. Written informed consent from children’s parents was obtained before participation in this study.

Methods

All participants in the current study were subjected to complete blood picture (CBC) and platelets count (Sysmix K-21 automatic cell counter. Japan).

5cc of venous blood was collected by sterile venipuncture. Each sample was divided into two parts: EDTA free part for ELISA, the other part was collected in EDTA containing blood collection tube and processed as whole blood within 24 h after venipuncture. Platelets count was done as part of the routine laboratory panel in the central laboratory of Benha University Hospital (Sysmix K-21 automatic cell counter. Japan). Quantification of reticulated platelets in EDTA containing blood collection was described later in Flow Cytometric Analysis. Sera samples from patients and controls were liquated and kept frozen at-20°C.

IL-16 was measured by a commercial ELISA (Quantikine, R&D Systems, Inc. USA). The detection
limits ranged from 2.7-13.4 pg/mL. ELISA procedures were done according to instructions of the manufacturers.

In brief, 100 μL of Assay Diluent RD1W was added to each well. 100 μL of standard, control, or sample per well were added then incubated for 2 hours at room temperature. Aspiration and wash were repeated for three times. 200 μL of Human IL-16 Conjugate was added to each well then incubated for 2 hours at room temperature. Aspiration and wash were repeated. 200 μL of Substrate Solution was added to each well then incubate for 30 minutes at room temperature. 50 μL of Stop Solution was added to each well. The optical density of each well was determined within 30 minutes, using a microplate reader set to 450 nm. Obtained optical density values, were converted into pg/mL by the Bio Rad ELISA data analysis software. All experiments were performed in duplicate and data represent mean values. The cut-off value was calculated as the mean absorbance value of the negative controls plus three standard deviations. A sample was considered positive when the absorbance of the two measurements was greater than 0.178.

Flow Cytometric Analysis

It was done at Benha Pediatrics Specialist Hospital.

Whole-blood, dual-labelling flow cytometric method was used. Direct, whole-blood double coverage was achieved using a monoclonal antiglycoprotein CD41 antibody (CD41-PE) for platelet identification and analysis of reticulated platelets (Youngest plateletes) by binding of thiazole orange (TO) (Retic-count(R) which is a fluorescent dye bind to mRNA content of platelets and allow their enumeration by Fluorescent activated cytometric (FACS) analysis.

10 μl of blood samples with EDTA as an anticoagulant, were centrifuged at 750 g for 5 minutes to obtain platelet-rich plasma. The platelets were fixed in 1% paraformaldehyde for 5 minutes at room temperature to minimize nonspecific staining and resuspended at 5x10⁷/mL in phosphate-buffered saline (PBS). Fifty microliters of this suspension was mixed and incubated with 10 μl phycoerythrin (PE) -anti-CD41-monoclonal antibody (CD-41 PE, DAKO, USA) at room temperature for 30 min. Cells were then washed twice in PBS. This suspension was then incubated with 1 mL of To (Retic-COUNT, Becton Dickinson, San Jose, CA, USA) at room temperature in the dark for 1 hour. Labeled platelets were analyzed on FACS-caliber using cell quest software (Becton Dickinson San Jose, CA, USA).

Live gating on platelet-sized events was performed using the distinctive forward light scatter (FSC) and vs side light scatter (SSC) profile of platelets (figure 1). Ten thousand events were measured for each sample. The second gating was performed using forward-scatter vs FL2 fluorescence, and the CD41 PE-tagged platelet population was selected. For phycoerythrin 580 nm filter is used. To exclude cell autofluorescence and instrument background, an unstained control sample was prepared simultaneously for each sample. TO-stained platelets with higher fluorescence than the 99% of the unstained control samples were considered to be reticulated platelets. The histogram for these gated populations were then obtained and the mean fluorescence intensity (MFI) were calculated using Cell Quest software which obtains the linear channel numbers, determines the average values for 10000 events, and then converts to mean log channel number.

Statistical Analysis

These data were tabulated, coded then analyzed using the computer program SPSS (Statistical package for social science) version 16. Results were expressed as mean and standard deviation (SD). parametric data were analysed with unpaired student’s t test were appropriate. Correlation between variable were analyzed using Spearman rank correlation. P<0.05 was considered significant and P<0.001 was considered highly significant.
Results

This study was conducted on 26 children with ITP and 12 healthy volunteers as control group. Patients’ and controls’ data were summarized in Table (1). The most frequent symptoms in ITP patients were petechia in 19 patients (73.1%). Ten children (38.5%) were admitted with epistaxis with petechia. There was history of blood transfusion for 7 children (26.9%). There were no differences between patients with regards to mean platelet count, age and sex. Platelets counts were significantly different between patients and controls ($P<0.001$) but no significant difference between patients and controls regarding the age.
Table 1. Demographic and clinical data of ITP patients and control groups.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Newly diagnosed ITP</th>
<th>Persistent ITP</th>
<th>Chronic ITP</th>
<th>Control</th>
<th>( P ) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years) (mean ±SD)</td>
<td>5.55 ± 2.278</td>
<td>5.45 ± 1.383</td>
<td>5.65 ± 2.224</td>
<td>6.79 ± 2.65</td>
<td>NS</td>
</tr>
<tr>
<td>Sex F/M</td>
<td>6/5</td>
<td>4/5</td>
<td>4/2</td>
<td>5/7</td>
<td></td>
</tr>
<tr>
<td>Platelets count (mm(^3)) (mean, range)</td>
<td>10430 (2500-50000)</td>
<td>23560 (4200-53000)</td>
<td>25370 (4600-5900)</td>
<td>240000 (150000-440000)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Bleeding manifestations</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Petechia</td>
<td>11</td>
<td>5</td>
<td>3</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Epistaxis</td>
<td>3</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Petechia and epistaxis</td>
<td>3</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Melena</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Hematuria</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>History of blood transfusion</td>
<td>-</td>
<td>3</td>
<td>4</td>
<td>-</td>
<td></td>
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</tbody>
</table>

Comparisons between each of the four groups were analysed by unpaired t test; comparisons among the four groups were also compared using one-way analysis of variance with adjustment for multiple comparisons. \( P<0.05 \) was considered statistically significant. NS: not significant.

The study revealed that all patients with ITP had markedly increased serum IL-16 (782.5±730.8, 284.2±309.5 and 159.2±142.6) in newly diagnosed, persistent and chronic ITP respectively that differed significantly from those of the healthy subjects (24.3±6.7) \((P<0.001)\) (Figure 2).

Figure 2. Comparison between serum IL-16 levels in different groups of patients with ITP and controls.
Our study also demonstrated that serum IL-16 levels were significantly elevated in newly diagnosed ITP compared to its levels in persistent ITP and chronic ITP ($P<0.01$, $P<0.001$) respectively. IL-16 levels were also significantly higher in persistent ITP compared to chronic ITP ($P<0.001$) Table 2 & 3.

Patients with newly diagnosed ITP had markedly increased positive CD41 platelets percent (20.1 ± 0.7) that differed significantly from those of the healthy subjects (9.4±2.4). It was also elevated in persistent and chronic groups (11.6±0.3 and 11.5±0.8) compared to the control group. However, these values were statistically significant in newly diagnosed group ($P<0.05$) and insignificant in persistent and chronic group ($P>0.05$) Table 2.

Percentage of positive CD41 platelets was 20.1±0.7 in newly diagnosed, ITP, 11.6±0.3 in persistent ITP and 11.5±0.8 in chronic ITP. These values were significantly different in newly diagnosed ITP compared to its levels in other two ITP groups ($P<0.05$) each but no significant difference in persistent ITP compared to chronic ITP ($P>0.05$) Table 3.

<table>
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<tr>
<th>Table 2. Comparison of serum IL-16 levels and platelet's count and CD41 expression between ITP patients and control groups.</th>
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<tbody>
<tr>
<td><strong>IL-16 (pg/ml)</strong></td>
</tr>
<tr>
<td>(mean ±SD)</td>
</tr>
<tr>
<td><strong>CD 41%</strong></td>
</tr>
</tbody>
</table>

$P1$ = newly diagnosed ITP vs control, $P2$ = chronic ITP vs control, $P3$ = chronic ITP vs control. $P>0.05$ is not significant (NS)

<table>
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<tr>
<th>Table 3. Comparison of serum level of IL-16 and percent CD 41 positive platelets among pediatric groups with ITP.</th>
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<tbody>
<tr>
<td><strong>IL-16(pg/ml)(mean ±SD)</strong></td>
</tr>
<tr>
<td>$P$ value</td>
</tr>
<tr>
<td><strong>CD 41%</strong></td>
</tr>
<tr>
<td>$P$ value</td>
</tr>
</tbody>
</table>

$P1$ = newly diagnosed ITP vs persistent ITP, $P2$ = newly diagnosed ITP vs chronic ITP, $P3$ = persistent ITP vs chronic ITP. $P>0.05$ is not significant (NS)

Histogram of TO labeled platelets from normal controls and ITP patients is presented in Figure 3. Labeled platelets from ITP patient groups showed more fluorescence intensity than those of the normal control group. MFI of the platelets were 57.59±12.22, 77±15.22, 92±16.11 and 105±25.83 in arbitrary fluorescence units for control, chronic, persistent and newly diagnosed ITP, respectively.

Negative correlation was found between platelets count and IL-16 level and CD41% in patients with ITP ($r=-0.248$, $P=0.028$, $r=0.274$, $P=0.025$) respectively figure 4 & 5.
Figure 3. Flow cytometric histogram of TO-labeled platelets. Platelets were stained with TO dye and their intensity was measured as MFI using a flow cytometer. M markers were set at the inflection points on the curve. The M1 indicates platelets that stained positive for TO in a representative healthy control and M2 in a representative ITP patient. MFI values were (23 and 89) respectively.

Figure 4. Correlation between platelets counts and serum IL-16 level in patients with ITP. Negative correlation was found between platelets counts and IL-16 level in patients with ITP ($r=0.248$, $P=0.028$).
Discussion

ITP is a common human autoimmune disease. It characterized by destructive thrombocytopenia due to the presence of auto antibodies with a normal or an increased number of bone marrow megakaryocytes (Kaplan et al., 2004). These autoantibodies opsonize platelets for splenic clearance. Therefore, the life spans of circulating platelets in ITP are reduced.

The disease is characterized by recurring episodes of severe thrombocytopenia and is associated with a bleeding diathesis. Treatment for ITP includes splenectomy, high-dose corticosteroids and intravenous immunoglobulin's (IVIg) (Stasi et al., 2008).

The present study demonstrated significant differences in serum level of IL-16 between newly diagnosed pediatric patients with ITP, persistent and chronic pediatric patients with ITP. This was in agreement with Jernâs et al., 2013, who found that plasma IL-16 was significantly higher in newly diagnosed than in chronic ITP. They also verify this finding in a second Chinese cohort of newly diagnosed ITP children and children with chronic ITP. The study also revealed that IL-16 was elevated in all patients groups compared to the control group. Richmond et al., 2014 explained that IL-16 has been a contributing factor in the orchestration of an immune response during autoimmune disease.

To the best of our knowledge no other relevant work regarding to the role of IL-16 in ITP has been done to compare our work with.

Gu et al., 2012, showed that serum IL-16 levels were elevated both in Hashimoto thyroiditis (HT) and higher in untreated Graves disease (GD) patients when compared healthy individuals, which were decreased after therapy in untreated GD patients. They concluded that IL-16 might be involved in the pathogenesis of GD and HT, and serum IL-16 levels in GD maybe a potential marker of disease activity and severity.

In a study done by Wu et al., 2011, they found that serum levels of total IgE, IL-16...
correlated with the SCORAD index in pediatric patients with atopic dermatitis. These serum parameters declined significantly after conventional treatment with clinical improvement. Thus, they postulated that they can serve as serum markers for monitoring disease activity in childhood atopic dermatitis.

Zak et al., 2010, reported that elevated levels of IL-16 with the lower blood content of the cytokine IL-4 were long before the development of DM type1 in children with normal blood glucose level in the presence of Langerhans islet autoantibodies (LIAA), which should be borne in mind while developing the immune mechanisms specifically directed against block, which participate by means of cytokines in beta-cell destruction, as well as methods for preventing the development of T1DM in subjects with LIAA. Meagher et al., 2010, also demonstrated that IL-16 production by leukocytes in islets of Langerhans augments the severity of insulitis during the onset of type 1 diabetes. Neutralization of IL-16 may represent a novel therapy for the prevention of type 1 diabetes.

Kimura et al., 2011, reported that IL-16-deficiency reduced graft inflammatory cell recruitment, and allograft inflammatory cytokine and chemokine production. Therefore, IL-16 neutralization may provide a potential target for novel therapeutic treatment for cardiac allograft rejection.

This study revealed that percentage of reticulated platelets was elevated in all patients groups compared to the control group, however, these values were statistically significant in newly diagnosed group and insignificant in persistent and chronic groups compared to control. This was in accordance with Giovanni et al., 2012, as regard newly diagnosed and persistent ITP as they found that the percentage of reticulated platelets were significantly higher in children with acute and those with chronic progression of ITP (equivalent to persistent ITP in our study) than in healthy subjects ($P<0.01$) and in contrast with them as regard chronic ITP ($P<0.001$). They stated that their finding probably mirror ongoing massive platelet destruction, do not, therefore, seem to have a prognostic meaning with regards to ITP progression.

Our results confirmed by observation of Rajantie et al., 2004, as regard of increase reticulated platelets count in persistent ITP who demonstrated that after 6 months of ITP (persistent stage) reticulated platelet counts increased in children with disease progression.

The present work was in contrast with Yildirmak et al., 2005, who found that platelet surface antigens were significantly decreased in both acute and chronic ITP when compared to the control group ($P=0.001$). Zhongguo et al., 2009, found that the expression of CD41 in acute ITP and the chronic ITP groups were significantly lower than those in the control group ($P<0.01$). The acute ITP group had lower expression of CD41 than the chronic ITP group ($P<0.01$). Jimin et al., 2008, explained that the numbers of antibody-binding sites of CD41 in the ITP patients were less than in the normal controls ($P<0.05$) by that the antibody-binding sites of CD41 may be occupied by autoantibodies and that the available binding sites may be decreased in ITP.

In study done by Yoshiyuki et al., 2001, they found that the percentage of reticulated platelets was markedly elevated in patients with ITP but normal or slightly elevated in patients with aplastic anemia or chemotherapy-induced thrombocytopenia. They reported the measurement of reticulated platelets percentage was an excellent method for discriminating between hyper destructive and hypo plastic thrombocytopenia.

Thomas et al., 2007, showed that patients with thrombocytopenic myelodysplastic
syndrome had normal reticulated platelets counts compared to healthy subjects. ITP patients had elevated reticulated platelets counts. They concluded that reticulated platelets counts had a diagnostic and prognostic value in patients with ITP and help to identify the complex causes of thrombocytopenia.

The present study demonstrated that serum level of IL-16 and reticulated platelets counts correlated inversely with platelets counts. Our results represent a remarkable contrast with those of Giovanni et al., 2012 who did not find a correlation between platelet count and reticulated platelets. They speculated that 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.

These results indicated that IL-16 may play an important role in activation of the immune response in ITP and suggested that the two markers (IL-16 and CD41 percentage) are altered in the different stages of pediatric ITP.

We conclude, that increased IL-16 and elevated reticulated platelets are observed in all forms of ITP. They seem to predict the clinical course of ITP, as IL-16 is significantly higher and reticulated platelets significantly elevated in the newly diagnosed ITP. Furthermore, IL-16 should be considered as a marker for discrimination between healthy children and those with ITP.

Further studies as well as new experimental designs are recommended to elucidate the role of IL-16 in pathogenesis, disease progression, diagnosis, and prognosis of ITP. IL-16 correlate with clinical manifestations of ITP, and may enable better prediction and/or directed therapy once confirmed in future studies.

References


10. Guidelines for the investigation and management of idiopathic thrombocytopenic purpura in adults,


