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

Athanasia Mouzaki, Maria Theodoropoulou, Ioannis Gianakopoulos, Vassiliki Vlahia, Maria-Christina Kyrtonis, and Alice Maniatis

Introduction

Idiopathic thrombocytopenic purpura (ITP) is an acquired autoimmune disorder characterized by the production of antibodies against antigens on the membranes of platelets, resulting in enhanced Fc-mediated destruction of the platelets by macrophages in the reticuloendothelial system. ITP is mainly classified into 2 forms, chronic ITP, typically an adult disease persisting for years, and acute ITP, a self-limited childhood disorder usually occurring within weeks after a viral infection. ITP is more common in children than in adults and approximately 40% of all patients are younger than 10 years. In children, both sexes are equally affected; in adults, women predominate 3:1. 1,6

Although autoreactive B lymphocytes secreting antiplatelet antibodies are considered as the primary immunologic defect in ITP, several T-lymphocyte abnormalities have also been described. Cell-mediated cytotoxicity against platelets has been demonstrated using lymphocytes and T-cell clones from patients with ITP. In addition, abnormalities have also been described in the analysis of T-cell subsets, mainly a decreased CD4 population and reversed CD4/CD8 ratios, higher numbers of CD45RA, and lower numbers of CD45RO T cells, all reminiscent of the association of HIV infection and thrombocytopenia as well as other autoimmune phenomena, such as systemic lupus erythematosus, the initial presentation of which may be ITP. 7-11

Administration of intravenous immunoglobulin G (IVIg) is the standard therapy used in recent years for ITP and is effective in a significant proportion of patients. 2,12 The proposed mechanisms of action of IVIg include the saturation of phagocytic Fc receptors or the neutralization of antiplatelet autoantibodies by anti-idiotypic antibodies in the preparations. 2 It has been reported that antibodies against interleukin 1 (IL-1) and IL-6 were detected in IVIg preparations. 3 Because IL-1α, associated with the cytoplasmic membrane of antigen-presenting cells, appears to be particularly important in the triggering of T lymphocytes, 14 its neutralization by anti–IL-1α antibodies contained in IVIg preparations may result in immunosuppression in vivo. In addition, IgG1 and IgG2 antibodies to IL-1α may trigger cytotoxic processes directed against both IL-1α-producing and IL-1α–responding cells, which may result in a rapid decrease in the number of circulating T and B cells. 14

Another possible effect of IVIg administration may be the masking of superantigen-binding sites on T cells, thus modulating the superantigen-induced cytokine production in T cells. 13 It has also been shown in vitro that IVIg down-regulates the synthesis of certain cytokines as well as cell-surface IL-2 receptor expression. 15 It has also been reported that transforming growth factor β (TGF-β) contained in varied quantities in IVIg preparations contributes to the therapeutic effect of IVIg in autoimmune diseases 17; this finding is disputed by others. 18

From the Laboratory Hematology and Transfusion Medicine and Department of Pediatrics, School of Medicine, Patras University, Patras, Greece, and Hematology Unit, Laikon Hospital, Athens, Greece.

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Reprints: Athanasia Mouzaki, Laboratory Hematology and Transfusion Medicine, School of Medicine, University of Patras, Patras GR-261.10, Greece; e-mail: mouzaki@med.upatras.gr.

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In recent years, numerous studies19-23 have shown that patients suffering from autoimmune diseases have polymerase chain reaction (RT-PCR) Reverse transcriptase (RT) have been described. Childhood ITP does not seem to fit the definition of an established autoimmune disease and although spontaneous remission occurs in the majority of children, it still remains impossible to predict at the time of diagnosis which child will develop an acute self-resolving disorder and which a chronic disorder.2,3 To this end, we studied the expression of a panel of Th1 and Th2 cytokine genes in a group of children who presented with ITP and assessed the effect of IVIg administration on the cytokine gene expression of the above patients. In addition, we performed follow-up studies in the same children 0.5 to 5 years later to investigate whether the primary findings can be of prognostic value. Although the data obtained are based on a small number of cases studied, it appears that the Th cytokine profile at presentation and after IVIg infusion can predict the clinical course of childhood ITP.

Patients, materials, and methods

Patients

Eighteen patients with acute ITP and 14 healthy children as controls were studied. All subjects presented to the Pediatric Department of the Patras University Hospital (PUH). Samples of heparinized blood (0.5-5 mL) were drawn from patients prior to any treatment and 24 hours after completion of IVIg administration, and from controls only once during a routine visit to PUH for minor problems. Samples were also collected at follow-up visits at least once for each patient. Informed consent was obtained from each participating patient (when old enough) and the patient’s parent or guardian. Human experimentation guidelines were submitted to and approved by the Human experimentation advisory committee of PUH. PUH abides by the Helsinki declaration on ethical principles for medical research involving human subjects.

IVIg

The IVIg preparation used was Sandoglobulin (Novartis, Basel, Switzerland). When reconstituted for therapeutic use it contained 50 mg/mL IgG, 25 to 35 mg/mL sucrose, 6 to 10 mg/mL glucose, and 40 to 100 mM NaCl. The IVIg preparation used was Sandoglobulin (Novartis, Basel, Switzerland). When reconstituted for therapeutic use it contained 50 mg/mL IgG, 25 to 35 mg/mL sucrose, 6 to 10 mg/mL glucose, and 40 to 100 mM NaCl.

Cell cultures

Heparinized venous blood was collected from the ITP patients at presentation, 24 hours after IVIg treatment, and at follow-up visits as well as from healthy pediatric controls. Peripheral blood mononuclear cells (PBMCs) were prepared by centrifugation over a Ficoll-Paque gradient (Pharmacia, Uppsala, Sweden). Plasma was collected and stored at −80°C. The cells (10⁶ PBMCs) were prepared immediately or cultured for 8 hours in RPMI 1640 culture medium (Gibco BRL, Gaithersburg, MD) containing 10% fetal calf serum (FCS; Gibco BRL) and other supplements as previously described,23 in the presence of 5 ng/mL phorbol myristate acetate (PMA) and 1 µM ionomycin (Sigma, St Louis, MO). The cells were counted using a Sysmex NE-8000 counter (Kyoto, Japan) and their viability was estimated by the trypan blue exclusion method as described previously.24

TGF-β1 ELISA

Determination of TGF-β1 levels in the plasma and also in different IVIg preparations was performed by an enzyme-linked immunosay (ELISA) as instructed by the manufacturer (Quantikine; R & D Systems, Minneapolis, MN).

Reverse transcriptase–polymerase chain reaction (RT-PCR)

Total cellular RNA isolated by the guanidinium thiocyanate–phenol–chloroform extraction procedure, as described by Chomczynski and Sacchi,25 was reverse transcribed (RT) after heat denaturation and annealing, with Random Hexamer (Promega, Madison, WI), in the presence of 200 U Superscript RT (Gibco BRL) and 0.5 mM of each deoxynucleotide (Promega), in 50 µL, for 1 hour at 37°C. Then 1 µL of the RT mixture was submitted to polymerase chain reaction (PCR), in a volume of 50 µL, in the presence of 150 µM of each deoxynucleotide, 2.5 U Taq polymerase (Gibco BRL), and 0.25 µM of the upstream and downstream primers (Institute of Molecular Biology, Crete, Greece). Each reaction was carried out with RNA extracted from 6.5 × 10⁶ viable PBMCs. The PCR products were run on 2% agarose gels and stained with ethidium bromide for UV light visualization and photography. For quantitative evaluation, the bands were scanned and the data analyzed using ImageTool V1.28 software (University of Texas Health Science Center, San Antonio, TX). The RT-PCR signal generated by β2-microglobulin (β2m) mRNA was chosen to estimate the amounts of cDNA obtained from different cell samples. Each RT-PCR included controls for RNA extraction (lysis buffer alone treated as a normal sample), RT (RT reagents without RNA), and PCR (PCR reagents without cDNA). The PCR primer pairs26-32 used in this study are shown in Table 1.

EMSAs

Electrophoretic mobility shift assays (EMSAs) were performed to assay for the presence and function of the lymphotropic transcription factors (TFs) nuclear factor-κB (NF-κB),33 activator protein-1 (AP-1)34, and nuclear factor of activated T cells (NFAT)35 as described previously36 with modifications. Briefly, the cellular membranes were broken by sonication on ice and a 3 to 4 × pellet volume of a buffer containing 5 mM HEPES (N-2-hydroxyethylpiperazine-n'-2-ethanesulfonic acid), pH 7.9, 26% glycerol, 1.5 mM MgCl2, 0.2 mM EDTA (ethylenediaminetetraacetic acid), 0.5 mM DTT (dichlorodiphenyltrichloroethane), 0.5 µM PMSF (phenylmethylsulfonyl fluoride), and 1 µg/mL leupeptin (all from Sigma) was added and then salt (KCl) adjusted to 300 mM to elute the proteins from chromatin. After an incubation of 30 minutes on ice, the cell lysates were centrifuged at 27 000g, for 60 minutes, at 4°C to sediment chromatin. Protein concentration was determined using the Bradford assay (Bio-Rad, Hercules, CA). As oligonucleotide probes we used the sequences AGTTGAGGGA TTTGGTCT for NFA T, GTGACTCAGGCCG for AP-1, and AAGAAAGTCACTT for NFAT.

Results

Patients

Table 2 shows the age, sex, and platelet counts of patients and controls. Table 3 shows clinical and laboratory parameters of

Table 1. Primers and conditions for the RT-PCR experiments performed in this study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence of primers (5’ → 3’)</th>
<th>T (°C)</th>
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<tr>
<td>β2m</td>
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T (°C) indicates annealing temperature; product, PCR product size (bp).
Table 3. Clinical and laboratory parameters of ITP patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
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<th>IV Ig (d)</th>
<th>PLT (24 h after IV Ig)</th>
<th>Time after IV Ig (d)</th>
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<tr>
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<td>3</td>
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<td>1</td>
<td>30</td>
<td>0.5</td>
<td>216 Relapsing ITP</td>
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Group 1 (P1-P12), patients with acute ITP followed by long-term remission; P8 and P9 received no treatment; group 2 (P13-P18), patients P13 to P16 with acute ITP followed by infrequent relapses triggered by viral infections; P17 and P18 had acute ITP followed by frequent episodes occurring within periods of less than 1 month, regardless of infection, requiring continuous monitoring. Only the times when blood samples were given for the present study are shown in the follow-up phase. PLT indicates number of platelets (× 10^9/L); IV Ig, 2 g/kg body weight (daily dose); NA, not applicable; time after, time in years after the first episode when blood sample was taken for the follow-up study.
The highest IFN-disease studied. At follow-up, 0.8 to 1.1 years later, they maintain recall patients. Before Rx indicates at presentation; after Rx, 24 hours after IVIg therapy; follow-up, than 1 month, regardless of infection, requiring continuous monitoring (black bars). P18 with infrequent relapses triggered by viral infection (gray bars) and ITP patients P17 and other bars); Panel B shows group 2 ITP patients P13 to P16 with the results were quantified and given as pixels/10² in the children with the chronic active disease.

They have a constant Th1-high-IFN-γ pattern in all phases of the disease studied. At follow-up, 0.8 to 1.1 years later, they maintain the highest IFN-γ levels of all patients with ITP.

Ex vivo IL-10 gene expression

On the whole, IL-10 gene expression (Figure 2) correlated negatively with disease activity (Figure 2, total). In group 1, it was highly expressed in the acute phase; its expression decreased after IVIg infusion and reached zero levels at follow-up.

In group 2, IL-10 expression was highly expressed at all times in the relapsing category with a transient decrease after IVIg infusion, whereas it was not expressed at any point of the study in the children with the chronic active disease.

TGF-β1 levels in sera and IVIg preparations

An ELISA was used to measure TGF-β1 levels in the plasma of the children with ITP before and 24 hours after IVIg treatment and at follow-up at the times indicated in Table 3 in pediatric controls and 15 randomly selected preparations of IVIg. The results are depicted in Figure 3 and show that TGF-β1 plasma levels increased immediately after treatment in the majority of the patients and the IVIg preparations tested contained negligible levels of TGF-β1 (mean = 1.14 ng/mL).

Absence of a transcriptional silencer in patients with ITP

The TFs AP-1, NF-κB, and NFAT regulate cytokine transcription.34,35,37 Of the 3 TFs, AP-1 and NF-κB are exclusively positive regulators, whereas NFAT TFs play a dual role. An NFAT-silencer (NFAT-S) down-regulates transcription and an NFAT-transcription activator (NFAT-A) up-regulates transcription. The silencer is only detectable in resting naive CD4 T cells and is lost in recently activated cells (effectors) and resting memory cells. The activator is detectable in activated and memory cells.24 It was thus suggested that the presence of a silencer contributes to the more stringent activation requirements of naive CD4 T cells, thus safeguarding against autoimmunity.24 To test this hypothesis, we performed EMSA experiments with AP-1, NF-κB, and NFAT probes and nuclear extracts from ex vivo or mitogenically stimulated peripheral blood lymphocytes (PBLs) from a healthy child and 2 children with ITP from group 1, in the acute phase and in remission. Phenotypic analysis performed in remission showed that these patients, like the healthy child, had over 50% resting naive CD4 T cells (data not shown). The results are shown in Figure 4. At presentation, the patients, in contrast to the healthy control, had AP-1, NF-κB, and NFAT-A activity in their ex vivo T cells and, similarly to the healthy control, in their mitogenically stimulated cells. In remission, none of these TFs were present in the nucleus of the patients’ ex vivo cells but were present in their stimulated cells, that is, the same pattern with that in the healthy control, with the exception that the NFAT-S was missing.

Discussion

Established autoimmune diseases have polarized Th1 and Th2 responses.19-23 Acute ITP is a childhood autoimmune disorder that either resolves after the first episode or develops into a relapsing form with rare episodes triggered by viral infections or, rarely, into a more serious chronic form that requires maintenance treatment.2,3,5 Studies performed with a selected population of chronic adult ITP patients with active disease observed neither a clear-cut Th1 nor a Th2 serum cytokine profile,39 whereas studies performed with a mixed population of patients with acute or chronic or

Figure 1. Ex-vivo Th1/Th2 cytokine gene expression profiles in patients with ITP versus healthy pediatric controls. PBMCs were isolated from the patients and controls and RNA was extracted immediately (ex vivo). RT-PCR was performed and the results were quantified and given as pixels/10² (SEM as described in “Patients, materials, and methods”). Panel A shows 14 healthy pediatric controls (left) and group 1 ITP patients P1 to P12 with first episode followed by stable remission (gray bars); Panel B shows group 2 ITP patients P13 to P16 with first episode followed by infrequent relapses triggered by viral infection (gray bars) and ITP patients P17 and P18 with first episode followed by frequent episodes occurring within periods of less than 1 month, regardless of infection, requiring continuous monitoring (black bars). Before Rx indicates at presentation; after Rx, 24 hours after IVIg therapy; follow-up, recall patients.

Figure 2. Expression of the IL-10 gene in ITP patients compared to pediatric controls. Pediatric controls, far left; total: results from all 18 patients. Group 1 indicates patients P1 to P12; group 2, patients P13 to P18; P13 to P16 patients with relapsing ITP; P17 and P18 patients with chronic ITP. Before Rx indicates at presentation; after Rx, 24 hours after IVIg therapy; follow-up, recall patients.

Figure 3. TGF-β1 levels in plasma of patients and controls and in IVIg preparations. TGF-β1 concentrations (mean values ± SEM) measured by ELISA in the plasma of the pediatric controls and of all the patients at presentation (before Rx), after IVIg treatment (after Rx), and at follow-up, and in different IVIg preparations.
chronic-complex ITP showed a Th0/Th1 pattern of T-cell activation.\textsuperscript{10}

We analyzed the Th cytokine expression patterns in 18 children with acute ITP, before and after IVIg administration, and at follow-up. In parallel, we determined the expression pattern of IL-10 and plasma levels of TGF-\(\beta\).

Compared to the healthy pediatric controls who expressed no Th cytokines in vivo, the majority of the children who presented with acute ITP were expressing IL-2, IFN-\(\gamma\) with or without IL-4 cytokine genes in vivo (72\% of the sample), suggesting an early CD4 Th0 and Th1 cell activation, in accordance with an earlier study.\textsuperscript{10} A minority of the patients (28\% of the sample) presented with no cytokine expression and these were 5 patients of group 1 who went into complete remission after IVIg infusion or with no treatment at all (2 patients).

Of the patients expressing Th1 or Th0/Th1, the first differentiating factor was their response to IVIg infusion. Those who expressed no cytokine or Th2 after IVIg treatment required no other therapy and went into long-term remission maintaining the 0 or Th2 pattern at follow-up. Those who maintained the Th1 or Th0/Th1 pattern after IVIg treatment were either refractory to IVIg or had a transient response to it. The differential effect of IVIg treatment in ITP patients probably reflects different degrees of imbalance of the immune system of patients,\textsuperscript{12} which could, in turn, reflect the relative frequency of autoreactive clones in the periphery. It has been shown that IVIg induces direct apoptosis of activated T and B lymphocytes and also monocytes/macrophages.\textsuperscript{40} We may thus hypothesize that the effectiveness of IVIg treatment correlates negatively with the severity of the disease.

The second differentiating factor was the relative intensity of IFN-\(\gamma\) gene expression: The patients with low IFN-\(\gamma\) expression at presentation (group 2) had either a transient response to IVIg (low IFN-\(\gamma\), Th1, or Th0/Th1) or were refractory to IVIg (high IFN-\(\gamma\), Th1). An important differentiating factor between the milder relapsing form and the aggressive chronic form of ITP in group 2 is the presence or absence of the anti-inflammatory cytokine IL-10. IL-10 provides a negative feedback mechanism that counteracts the activation of Th1 cells and monocytes/macrophages,\textsuperscript{23,41} so the complete absence of IL-10--expressing cells in the peripheral blood of patients P17 and P18 probably contributed to the enhanced Th1 cytokine synthesis by the autoreactive T-cell clones, which, in turn, inhibits IL-4 synthesis in these patients.

Based on the above, we propose the model depicted in Figure 5. Multicenter studies of children presenting with ITP (RT-PCR/cytokine gene expression) and receiving none or IVIg-only treatment, and followed for at least 6 months, will test the validity of our pilot study.

The TGF-\(\beta\) levels in the IVIg preparations tested were negligible, thus appearing inadequate to influence cytokine expression in the patients. The very low plasma TGF-\(\beta\) concentrations at the time of disease onset increased after IVIg treatment. We may assume that the low TGF-\(\beta\) levels during active disease leave the autoantibody production against autologous platelets uncontrolled.\textsuperscript{42} In addition, because TGF-\(\beta\) promotes thrombopoietin production,\textsuperscript{43} which, in turn, leads to increased platelet production,\textsuperscript{44} low TGF-\(\beta\) levels may also account directly for low platelet numbers during active disease. The relative increase of TGF-\(\beta\) levels in the patients after treatment and in remission may mediate a bystander immune suppression,\textsuperscript{39,45} which may be adequate for the majority of the patients who recover or have rare episodes of thrombocytopenia but inadequate for the patients with chronic active disease.

A noteworthy aspect of this study is the EMSA finding (Figure 4) that the NFAT transcriptional silencer was absent from the nucleus of the ex vivo T cells of the 2 patients tested who were in remission. The positive TFs AP-1 and NF-\(\kappa\)B were not active in these cells, indicating that the cells were not in vivo activated, whereas all positive factors including NFAT-A were activated in the in vitro–stimulated cells indicating that the cells were not anergic.\textsuperscript{45,46} Acquisition of the NFAT-S\textsuperscript{24,36,38} could play a role in

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The patients with high expression of IFN-\(\gamma\) at presentation (group 2) had either a transient response to IVIg (low IFN-\(\gamma\), Th1, or Th0/Th1) or were refractory to IVIg (high IFN-\(\gamma\), Th1). An important differentiating factor between the milder relapsing form and the aggressive chronic form of ITP in group 2 is the presence or absence of the anti-inflammatory cytokine IL-10. IL-10 provides a negative feedback mechanism that counteracts the activation of Th1 cells and monocytes/macrophages,\textsuperscript{23,41} so the complete absence of IL-10--expressing cells in the peripheral blood of patients P17 and P18 probably contributed to the enhanced Th1 cytokine synthesis by the autoreactive T-cell clones, which, in turn, inhibits IL-4 synthesis in these patients.

Based on the above, we propose the model depicted in Figure 5. Multicenter studies of children presenting with ITP (RT-PCR/cytokine gene expression) and receiving none or IVIg-only treatment, and followed for at least 6 months, will test the validity of our pilot study.

The TGF-\(\beta\) levels in the IVIg preparations tested were negligible, thus appearing inadequate to influence cytokine expression in the patients. The very low plasma TGF-\(\beta\) concentrations at the time of disease onset increased after IVIg treatment. We may assume that the low TGF-\(\beta\) levels during active disease leave the autoantibody production against autologous platelets uncontrolled.\textsuperscript{42} In addition, because TGF-\(\beta\) promotes thrombopoietin production,\textsuperscript{43} which, in turn, leads to increased platelet production,\textsuperscript{44} low TGF-\(\beta\) levels may also account directly for low platelet numbers during active disease. The relative increase of TGF-\(\beta\) levels in the patients after treatment and in remission may mediate a bystander immune suppression,\textsuperscript{39,45} which may be adequate for the majority of the patients who recover or have rare episodes of thrombocytopenia but inadequate for the patients with chronic active disease.

A noteworthy aspect of this study is the EMSA finding (Figure 4) that the NFAT transcriptional silencer was absent from the nucleus of the ex vivo T cells of the 2 patients tested who were in remission. The positive TFs AP-1 and NF-\(\kappa\)B were not active in these cells, indicating that the cells were not in vivo activated, whereas all positive factors including NFAT-A were activated in the in vitro–stimulated cells indicating that the cells were not anergic.\textsuperscript{45,46} Acquisition of the NFAT-S\textsuperscript{24,36,38} could play a role in
converting proliferating precursors into resting cells during T-cell maturation. We hypothesize that an error at this stage of lymphocyte maturation causes a failure in the acquisition of the NFAT-S by some otherwise mature thymocytes and results in an increased number in the periphery of naïve T cells, which are more prone to develop into autoreactive clones on antigenic stimulation.

Noted added in proof. Since this manuscript was submitted for publication a year has passed during which the patients included in this study were monitored, and their follow-up diagnosis remains the same.

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References
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

Athanasia Mouzaki, Maria Theodoropoulou, Ioannis Gianakopulos, Vassiliki Vlaha, Maria-Christina Kyrtonis and Alice Maniatis