

Phenotypic Analysis of Bone Marrow Lymphocytes From Children With Acute Thrombocytopenic Purpura

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Hematogones are benign immature B cells that commonly populate the bone marrow of children. Their presence has been noted to interfere with the flow-cytometric analysis of acute lymphoblastic leukemia (ALL), because their immunophenotype is similar to B-precursor cell lymphoblasts. Immune-mediated thrombocytopenia is a clinical condition characterized by increased platelet destruction due to sensitization of platelets by autoantibodies. The aim of this study was to determine the incidence and clinical impact of bone marrow hematogones in cases of acute immune thrombocytopenic purpura (ITP) among children. This was done by immunophenotyping of bone marrow lymphocytes of ITP cases and controls and follow up of cases. This study was done on 25 cases of ITP, 12 females and 13 males, their age ranged from 2 to 13 years. A control group was included in the study, 15 cases of apparently healthy children with matching age and sex taken from among bone marrow donors. Cases and controls were subjected to bone marrow lymphocyte immunophenotyping with flow-cytometry to verify the presence of hematogones. A statistically significant increase in the percentage of hematogones was demonstrated in their bone marrows. An increased percentage of CD10⁺ lymphocytes was demonstrated; with a mean of 18±15.2%, CD19⁺ with a mean of 27±16.3% and CD34⁺ with a mean of 3.7±3.2%. No correlation was found between the percentage of hematogones and peripheral platelet count or bone marrow lymphocytic count. In conclusion, there is an increase in the bone marrow hematogones in ITP cases in comparison to normal controls. This could be the sequence of an immunological response to the cause which determined the disease, or the regeneration of the stem cell compartment following transient damage.

Immune thrombocytopenic purpura (ITP) of childhood is an acquired, benign transient immune mediated thrombolytic condition. There are two criteria for making this diagnosis: thrombocytopenia with an otherwise normal complete blood count & no clinically apparent associated condition that may cause thrombocytopenia. ITP is a diagnosis of exclusion. ITP is caused by autoantibodies that are directed to platelet membrane antigens, primarily 2B and 3B and to a lesser extent, 1B and glycoprotein 9. Typical characteristics in ITP patients include; thrombocytopenia, in a bone marrow examination we would expect to find morphologically normal megakaryocytes in normal to increased numbers. The peak age among children developing ITP is 2 – 5 years, with no predilection between boys and girls. The sensitizing event is usually obvious & is classically a virus infection (George, 1996).

Bone marrows of children with ITP were reported by several workers to show an increase in benign B-cell precursors, also known as hematogones (Cornelius, 1991). Hematogones were first described in the 1930s as lymphoid appearing cells in sternal marrow aspirates. Most commonly observed in young children comprising up to 21% of marrow cells in normal infants (Vogel, 1939).

Hematogones may exhibit a spectrum of size and cytologic features that bridge those of mature lymphocytes and neoplastic lymphoblasts. They vary from 10-20 microns in diameter. Their nucleus is round or oval and sometimes exhibit one or more indentations or shallow clefts. Nuclear chromatin condensed & homogenous. Nucleoli are absent or indistinct. Hematogones have scanty cytoplasm, devoid of granules. Hematogone populations exhibit a complex spectrum of antigen expression that defines the normal evolution of B-lineage

precursors. The pattern of sequence and intensity of antigen expression is virtually identical in all people. The earliest recognizable B-lineage precursors express TdT and the progenitor cell marker CD34 in combination with CD38, CD19, high levels of CD10 and low levels of CD22; they lack CD20 (stage I). There is a stage where CD10/CD19 are co-expressed while kappa and lambda are completely negative; probably this is stage II hematogones, These progress to the next stages by down-regulating TdT and CD34 completely and CD10 partially, prior to progressive up-regulation of CD20. CD22 is also increased slightly as CD20 is up regulated (stage 3). Lastly CD10 is down-regulated completely. The cells in which CD10 is completely down regulated are considered mature B-lymphocytes (Wright, 2002). Depending on the stage of maturation of hematogones, markers could be: CD10, CD19, CD34 positive cells, CD10, CD19 positive cells or CD19, CD22 positive ones. That is why there is a different range of morphology from very immature (blast like) to more mature forms. Despite the frequency of hematogones, it is difficult to determine normal reference ranges for them, yet 5% or more is generally considered increased (Caldwell, 1991). It has become clear from the experience of several investigators that hematogones are found in highest numbers in marrow from infants and young children and decline significantly with increasing age. The percentage of marrow hematogones may fluctuate with disease status or persistent elevations may occur. They may be particularly abundant in regenerating marrow following chemotherapy or bone marrow transplantation as well as in patients with autoimmune or congenital cytopenias, hereditary spherocytosis and amegakaryocytic thrombocytopenia (Vandersteenhoven, 1993). On the other hand some non-hematological disorders are associated with increased hematogones; regenerative bone marrow after

viral injury, retinoblastoma, Gaucher disease and Human immunodeficiency virus (HIV). In several of these conditions hematogones may potentially cause diagnostic problems because of the morphologic and immunophenotypic features they commonly share with neoplastic B cell precursor lymphoblasts (D Arena, 2001). We studied the immunophenotype of bone marrow lymphocytes from ITP patients and controls, using a panel of monoclonal antibodies i.e. CD10, CD19 & CD34. Our aim was to determine the percentage and the clinical impact of bone marrow hematogones (CD10, 19, 34 positive lymphocytes) on the outcome of these patients.

Material and Methods

This study was conducted on 25 cases of newly diagnosed acute immune thrombocytopenic purpura (ITP) in children admitted to the children hospital. Their age ranged from 2 to 13 years. Fifteen apparently healthy children were included in the study selected from among bone marrow donors after parents consent. The diagnosis of ITP was made according to established criteria (George, 1996). In particular, all patients showed cutaneous hemorrhages of varying degree. All patients were subjected to history taking especially for a previous viral infection and for drug intake. A complete blood picture was done to all cases and controls to exclude other causes of thrombocytopenia e.g. acute leukemia. Bone marrow aspiration was done for all cases to verify the diagnosis & to get an EDTA sample for immunophenotyping.

Bone marrow flow cytometric analysis

Bone marrow cells were purified using ammonium chloride lysing solution; 0.037gm/L sodium EDTA, 1 gm/L potassium bicarbonate and 8.3 gm/L ammonium chloride (BDH). 5ml lysing solution are added to 1×10^6 cells, centrifuged for 5 minutes at 2000 rpm, cells were then washed twice using phosphate buffered saline (PBS). For each case three tubes were prepared, suitable volumes of the cells were added in each tube. In the first tube isotypic control was added. In the second 10 μ l of anti-CD10 FITC were added and anti-CD34 PE (phycoerytherin). In the third; 10 μ l of anti-CD19-FITC and anti-CD22 PE. These markers were assessed in combinations of FITC and PE labeled antibodies. The tubes were incubated for ten minutes, washed twice with PBS and were ready for

flowcytometry. Acquisition and analysis were done on a BD FACS Caliber (Becton Dickinson) and a cellQuest program. Ten thousand cells were acquired from each tube; the percent positive cells and the mean fluorescence intensity (MFI) were recorded.

Results

All patients studied presented with purpura and bruising with three presenting with epistaxis (12%), three with bleeding gums (12%), two patients presented with pallor (8%), another two presented with fever (8%). Assuming a possible correlation between the main clinical and hematological parameters, no differences were found in terms of age, absolute peripheral lymphocyte count, platelet count, bone marrow lymphocytes, number of previous fever episodes & percentage of hematogones. Immunophenotyping of bone marrow lymphocytes was done using our

panel of antibodies, mature B cells had mature markers; 19, 22, kappa and lambda whereas hematogones gave different stages of maturation; CD34, 19, 10 positive, CD19, 10 positive or CD19, 22. Bone marrow lymphocytes of ITP patients showed an increased percentage of CD10 positive cells with a mean of 18 ± 15.2 %, whereas among controls the percentage was 2 ± 1.8 %, this difference was statistically significant. The percentage of CD19 positivity was 27 ± 16.3 % among ITP cases and 1.13 ± 1.05 % among controls. This difference was also statistically significant. The same was found for CD34, where ITP cases gave a positivity level of 3.7 ± 3.2 %, while controls gave a level of 1.34 ± 1.21 %. This difference was significant as well (table 3).

Table 1. Phenotypic analysis in children with immune thrombocytopenic purpura.

Case No	CD10 %	CD19 %	CD34 %	BM lymphocytes %	Initial platelet count/cmm
1	4	16	2	21	30,000
2	19.2	35	1.8	26	21,000
3	18	40	2.6	42	20,000
4	2	11	1.4	25	23,000
5	8.8	14.3	1.08	2.6	19,000
6	2.8	5.6	2.8	18	30,000
7	11.8	35	4.1	23	30,000
8	12.9	16.5	2.1	36	18,000
9	30	6.7	0.79	24	17,000
10	5	32	1	22	20,000
11	8.7	18.7	14.8	25	29,000
12	26.4	32.4	5.9	8	26,000
13	33	29.6	0.5	21	40,000
14	20.9	36	6.65	20	35,000
15	55	60	7.3	27	39,000
16	19	23	2.8	25	24,000
17	13	26	1.4	22	20,000
18	12	13	0	15	35,000
19	18	12	5	21	30,000
20	31	12.3	5.4	27	25,000
21	0.8	7.6	2.2	20	35,000
22	1.3	59	6	18	12,000
23	61.4	46.2	2.6	17	18,000
24	17	31	7	20	33,000
25	20	57	5.2	25	36,000
Mean	18.08	27.03	3.7		

Table 2. Phenotypic analysis of controls.

Case No	CD10%	CD19%	CD34%
1	3.1	2.8	2.9
2	2.36	1.9	2.8
3	1.02	0.37	0.57
4	1.07	0.38	0.33
5	4.8	3.18	4.3
6	6.86	2.76	0.98
7	0.41	0.11	0.32
8	0.79	0.69	0.9
9	3	0.97	1.02
10	3.38	1.7	2.66
11	1.59	0.6	1.16
12	0.74	0.42	0.75
13	0.6	0.23	0.45
14	0.87	0.79	0.71
15	0.41	0.11	0.32
Mean	2.06	1.13	1.34

Table 3. Immunophenotyping of bone marrow lymphocytes of ITP patients & controls.

Immunophenotyping of BM lymphocytes	ITP cases %	Controls %	t test
CD10 percent	18 ± 15.2	2 ± 1.8	4.02 *
CD19 percent	27 ± 16.3	1.13 ± 1.05	6.07 *
CD34 percent	3.7 ± 3.2	1.34 ± 1.21	2 *

* Significance: $p < 0.01$

Discussion

Our experience, carried out on 25 consecutive children affected with ITP, compared to fifteen apparently normal children prepared to be bone marrow donors for siblings, confirmed that the incidence of the immature B phenotype, in accordance with other authors (Vincenzo, 1997) is increased. The CD10 antigen, together with CD19 & CD34, normally present on the lymphoid cells of fetal bone marrow, persists in considerable amounts in the bone marrow of normal children, decreases with advancing age and disappears almost entirely in adults (Caldwell, 1991; Loken, 1987). Typically, hematogone populations consist primarily of mid-stage B-cells with a lower proportion of early-stage cells or blasts and a few mature B cells. By flowcytometry, hematogones have a well-described immunophenotyping, characterized by variable expression of CD45 and strong expression of CD10 and CD19 (B lineage

markers). Only a few of these cells express the early blastic stage antigens, such as CD34 or TdT, or the immature markers CD20 and surface immunoglobulin. In common usage, the early and mid-stage B cells are termed hematogones (Rimsza, 2000; Wells, 1998). It was reported that in infants less than 2 years of age, hematogones averaged 9 %, by 2 – 5 years the percent dropped to 3.9 % and in patients more than 50 years of age, the average was less than 1 % (McKenna, 2001). In the study done by Rimsza et al, 2004, it was found that infants less than one week of age had a low percentage of bone marrow hematogones as compared to over 25 % in infants at 19 weeks of age. These findings support the hypothesis that the change in bone marrow hematopoietic activity resulting in increased lymphocyte percentages is triggered by birth and presumably antigen exposure rather than by a specific gestational age (Calhoun, 2000). These data suggest that a

mechanism involving immuno-stimulation of the lymphoid population probably underlies the increased proportion of lymphocytes expressing the immature B phenotype; hematogones, as demonstrated in regenerating bone marrow cured of ALL and after autologous bone marrow transplantation in acute myeloid leukemia (Smedmyr, 1991). We cannot exclude that an unknown viral or drug injury might be the cause which promotes an immunological response leading to the expression of the immature B phenotype. Alternatively, this phenomenon could be the result of the regeneration of the stem cell compartment after transient damage involving the platelet compartment. In general the presence of hematogones in the bone marrow of ITP and/or other benign conditions, may potentially cause diagnostic problems because of the morphologic and immunophenotypic features they commonly share with neoplastic B cell precursor lymphoblasts. Many of the circumstances in which hematogones are markedly increased are associated with clinical manifestations similar to those of ALL and lymphoblastic lymphoma e.g. blood cytopenias (Weir, 1999; Rego, 1998). In our study no relationship was found between patient age, sex and clinical presentation and percentage of hematogones. Hematogone populations always express a continuous and complete maturation spectrum and lack asynchronous or aberrant antigen expression (McKenna, 2001). On the other hand neoplastic lymphoblasts exhibit incomplete maturation and immunophenotypic asynchrony and aberrancy that deviates from the spectrum of antigen expression of normal stages of maturation (Borowitz, 1990). Only rare cases of precursor B ALL and lymphoblastic lymphoma express an immunophenotype that would be considered synchronous with a normal stage of B lymphocyte differentiation (Hurwitz, 1992). In the present study the highest percentage of positivity was found with CD19

(27 ± 16.3 %) and the lowest with CD34 (3.7 ± 3.2 %), this is expected as the commonest stage of B precursors in these conditions are stage 2 & 3 which are negative for CD34, the same was demonstrated by Rimsza 2000. None of our ITP cases developed ALL after a six months follow up period. In a study conducted by Vincenzo et al 1997; none of their cases developed lymphoproliferative malignancies after a follow up of over two years.

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