Introduction

Thrombocytopenia is a decrease in platelet count more than two standard deviation below the mean of the general population, that is, less than $150x10^9$/L ($150.000/mm^3$) in adults, children, and neonates. Mild (100 to $150x10^9$/L) and moderate (50 to $100x10^9$L) thrombocytopenia are rarely associated with clinical bleeding without trauma, while spontaneous bleeding can appear when the platelet count falls to 20-$30x10^9$/L ***(Murphy et al., 1999)***.

Thrombocytopenias can be generally divided into states of elevated platelet consumption, which are associated with shortened platelet mean life span, and states of decreased platelet production ***(Stockham and Scott, 2002)***.

Diagnostic methods to distinguish between these conditions have been limited, and bone marrow needle biopsy has been regarded as the gold standard ***(Thomas-Kaskel et al., 2007).***

Reticulated platelets are released into the peripheral blood following megakaryocytic fragmentation. Newly formed platelets, although anucleated, contain some rough endoplasmic reticulum and messenger RNA (mRNA) and are also able to synthesize small amounts of protein ***(Kieffer et al., 1987).*** Their measurement has considerable clinical utility for monitoring thrombopoiesis and platelet turn over ***(Harrison et al., 1997)*** as

the mRNA is unstable and degrades within 24 hours in animal models ***(Ault and Knowles, 1995).***

This RNA can be measured using a variety of dyes. Reticulated platelet can, therefore, potentially be quantified flow cytometrically using any fluorescent dye that binds RNA. The reticulated platelet can then be distinguished from the mature platelet that has not taken up the dye. ***Kienast and Schimitz (1990)*** developed a flowcytometric analysis of thiazole orange uptake of platelets in thrombocytopenic disorders and studied a variety of clinical conditions.

Under conditions of thrombocytopenia, platelet RNA content correlated directly with megakaryocyte activity. Patients with low megakaryocyte activity have no RNA elevation in their platelets while those with increased megakaryocyte activity have significantly elevated RNA-stained platelets. This offers the ability to determine whether thrombocytopenia is due to marrow failure or to increased peripheral destruction/ loss, thus avoiding the need for bone marrow examination ***(Briggs et al., 2004).***

Aim of the Study

Objectives of this work are:

1- To evaluate the diagnostic usefulness of reticulated platelets in different types of thrombocytopenia, to find out their clinical significance and to try to correlate reticulated platelets with response to therapy.

2- Try to determine the prognostic value of reticulated platelets in different types of thrombocytopenia.

Megakaryopoiesis and Thrombopoiesis

Hematopoiesis is a complex process that takes place in the yolk sac in the first few weeks then occurs in liver and spleen in the period from 6 weeks until 6-7 months of life. Liver and spleen continue to produce blood cells until 2 weeks after birth ***(Stohlawetz et al., 2001)***. After birth, the major site of hematopoietic activity shifts gradually from the liver and spleen to the bone marrow cavities of nearly all bones of the axial and appendicular skeleton. By early adulthood, the long bones no longer bear red marrow and the primary sites of red marrow are confined to the sternum, ribs, vertebrae and pelvis ***(Emerson, 2002)***.

Hematopoietic micro environment is composed of stromal cells such as fibroblast, macrophages, endothelial cells, adipocytes and accessory cells such as T-lymphocytes and monocytes, which are surrounded by various extracellular matrix and cytokines ***(Nagahisa et al., 1998)***.

Megakaryopoiesis is proliferation and maturation of platelet precursors while thrombopoiesis is production of platelets ***(Gewirtz and Ponez, 2000)***. Mechanisms of thrombopoiesis are of considerable interest in hematology and cell biology, in part because of the variety of human thrombocytopenia syndromes and because megakaryocyte (MK) differentiation encompasses many unusual attribute.

Mature MKs are polyploid cells that assemble a unique set of organelles, including alpha granules, dense bodies, and an extensive system of internal membranes. The cellular mechanisms of endomitosis in MKs have been clarified recently ***(Roy et al., 2001)***, and genetic studies are rapidly bringing the molecular basis of MK organelle biogenesis into sharper focus ***(Detter et al., 2000).***

I. Development:

Hematopoietic stem cells (HSC) can self replicate and differentiate into hematopoietic progenitor cells (HPCs). Like the other hematopoietic cells of the bone marrow, megakaryocytes are derived from undifferentiated pluripotent hematopoietic stem cells ***(Gewirtz and Ponez, 2000)***. The stem cells differentiate into committed megakaryocytes progenitor, the megakaryocyte mature by polyploidization and cytoplasmic maturation (megakaryopoiesis), and finally the mature megakaryocyte releases a number of platelets ***(Nagahisa et al., 1998)***.

Megakaryocyte development is artificially divided into three stages: progenitor cells, immature megakaryocyte (promegakaryoblast) and mature megakaryocyte. The first developmental compartment consists of progenitor cells and is entered upon stem cell commitment to the megakaryocyte lineage ***(Gewirtz and Ponez, 2000)***. In the megakaryocyte lineage, progenitors are of three types, defined by a

combination of their physical properties. The least mature progenitor cell is the burst-forming-unit megakaryocyte (BFU-MK) ***(Long et al., 1985)*** that proliferates into colony-forming-unit-megakaryocyte (CFU-MK) ***(Long, 1989)***. In vitro the BFU-MK has a high proliferation potential, generating 100-500 megakaryocytes per cell, in some five to seven replicative events ***(Long et al., 1985)***.

The BFU-MK appears to be sensitive to a different set of cytokines and hematopoietic growth factors that may up or down-regulate megakaryopoiesis. The major growth factor that derives megakaryocyte development is thrombopoietin (TPO). Other cytokines and growth factors, such as IL-3, IL-6, IL-11, erythropoietin and CSF-GM also appear to actively modulate the effect of TPO on megakaryocyte development ***(Brandt, 2002)***.

Colony-forming-unit megakaryocyte (CFU-MK) is more mature colony which was the first identified megakaryocyte progenitor cell ***(Gewirtz and Ponez, 2000)***. The CFU-MK cell has a restricted proliferative potential, generating only 4-32 megakaryocytes ***(William and Jackson, 1982)***.

The second development compartment consists of the immediate offspring of the megakaryocyte progenitor cell known as megakaryoblast as well as light microscopy-recognizable megakaryocyte. Megakaryoblasts are small mononuclear and express platelet specific phenotypic markers,

as platelet peroxidase, platelet glycoprotein IIb/IIIa and Von Willibrand Factor (VWF) ***(Rabllino et al., 1981)***.

Megakaryoblasts are not morphologically recognizable as megakaryocytes. They are thought to represent a transitional stage of development in megakaryopoiesis, bridging the gap between progenitor cells and morphologically identifiable megakaryocytes ***(Young and Weiss, 1987)***. They are endomitotic and contain an intermediate DNA ***(Long et al., 1982)***. Megakaryoblasts are the first cells to increase in number after induction of thrombocytopenia and to decrease under conditions of thrombocytosis ***(Long and Henny, 1979)***.

II. Platelet production and Release:

Megakaryocytopoiesis is a unique process, which leads to platelet production. It has two unique characteristics. First, the megakaryocyte (MK), the direct platelet precursor, is a polyploidy cell. This polyploidization occurs by a process called endomitosis. At the end of polyploidization, MKs complete their cytoplasmic maturation to finally shed platelets. Second, platelets are anucleate cells formed by the fragmentation of the MK cytoplasm ***(Botton et al., 2002)***.

There are three different proposed mechanisms of platelets production from megakaryocytes:

1. It was believed that demarcation membranes, internal membranes of the MK, determine platelet territories

corresponding to future platelets, which would be liberated via cytoplasmic fragmentation ***(Zucker-Franklin and Petursson, 1984)***.

2. **Proplatelet model:** Megakaryocytes send pseudopodia out into the bone marrow sinusoids and then platelets and proplatelets bud off. Proplatelets are elongated strands of megakaryocyte cytoplasm that are larger than normal platelets and later fragment into a number of platelets ***(Saito et al., 2005)***.

3. **Pulmonary platelets production model**: Intact mega-karyocytes reach the pulmonary capillaries and they release platelets by fragmentation of their cytoplasm at this site. Ninty eight percent of megakaryocytes leaving the lung are devoid of cytoplasm ***(Zucker-Franklin, 2000)***.

III. Platelet Kinetics:

In average, each megakaryocyte produces about 1000 to 5000 platelets. The maturation period of megakaryocytes is about 5 days ***(Turgeon, 1999)***. Initially, platelets enter the spleen, where they remain for 2 days. Following this period, platelets are in either the circulating blood or the active splenic pool. At all times, approximately two third of the total number of platelets are in the systemic circulation, while the remaining one third exists as a pool of platelets in the spleen that freely

exchange with the general circulation ***(Stenberg and Hill, 1999)***.

The life span of mature platelets is 9±1 day. At the end of their life span, platelets are phagocytosed in the liver and spleen and other tissues of the mononuclear phagocytic system ***(Turgeon,1999***). Platelets circulate as lenticular pieces of membrane-bound cytoplasm measuring 2-4 μm in diameter and having an average volume of 6-8 femtoliter. These measurements are important because in clinical situations, platelet number may not reflect total platelet volume ***(Ronald et al., 1999).***

Thrombocytopenia: The normal platelet count is 150-450 x 10^9/L. Thrombocytopenia refers to a reduction in platelet count to <150 x 10^9/L. Causes of thrombocytopenia includes: (1) decreased production on either a congenital or an acquired basis; (2) sequestration of the platelets within an enlarged spleen or other organ; and (3) increased destruction of normally synthesized platelets on either an immune or a nonimmune basis **(Nathan et al., 2003).**

Table (1) shows the differential diagnosis of thrombocytopenia in children and adolescents.

Table (1): Differential diagnosis of thrombocytopenia in children and adolescents.

Destructive Thrombocytopenia

Primary Platelet Consumption Syndromes

- Immune thrombocytopenias
  - Acute and chronic ITP.
  - Autoimmune diseases with chronic ITP as a manifestation.
    - Cyclic thrombocytopenia.
    - Autoimmune lymphoproliferative syndrome and its variants.
    - Systemic lupus erythematosus.
    - Evans syndrome.
    - Antiphospholipid antibody syndrome.
    - Neoplasia-associated immune thrombocytopenia.
  - Thrombocytopenia associated with HIV.
  - Neonatal immune thrombocytopenia.
    - Alloimmune.
    - Autoimmune (e.g., maternal ITP).
  - Drug-induced immune thrombocytopenia (including heparin-induced thrombocytopenia).
  - Post-transfusion pulpura.
  - Post-transplant thrombocytopenia.
- Nonimmune thrombocytopenias
  - Thrombocytopenia of infection.
    - Bacteria of fungemia.
    - Viral infection.
    - Protozoan.
- Thrombotic microangiopathic disorders
  - Hemolytic-uremic syndrome.
  - Thrombotic thrombocytopenic purpura.
  - Drug-induced.
- Platelets in contact with foreign material.
- Congenital heart disease.
- Drug-induced via direct platelet effects (ristocetin, protamine).
- Type 2B VWD or platelet-type VWD.

Continued…

Combined Platelet and Fibrinogen Consumption Syndrome

Disseminated intravascular coagulation .

Kasabach-Merrit syndrome.

Virus-associated hemophagocytic syndrome.

Impaired Platelet Production

Hereditary disorders

Acquired disorders

Aplastic anemia

Myelodysplastic syndrome

Marrow infiltrative process

Osteopetrosis

Nutritional deficiency states (iron, folate, vitamin B_{12}, anorexia nervosa)

Drug- or radiation-induced thrombocytopenia

Neonatal hypoxia or placental insufficiency

Sequestration

Hypersplenism

Hypothermia

Burns

(Nathan et al., 2003)

Reticulated Platelets

The name “reticulocyte” has historically been used to describe young erythrocytes that were originally stained with supravital dyes. These assays provided a simple means of assessing the erythropoietic activity of the bone marrow ***(Gyongyossy-Issa et al., 2003)***.

The platelet analogue for reticulocytes, “Reticulated platelets” was first described by ***Ingram*** and ***Coopersmith*** in ***1969***. By induction of acute blood loss in beagle dogs, a new population of platelets appeared that were shown to contain coarse and punctuate condensations when stained with New Methylene Blue (NMB). They were thought to be analogous to red cell reticulocytes which were stained in parallel. Flowcytometric/automated analysis of red cell reticulocytes is now becoming increasingly popular within hematology laboratories.

The first flowcytometric method for reticulated platelets was published in 1990 ***(Kienast and Schmitz, 1990)***, and has led to a number of groups using the assay primarily as a research tool.

During the 1990’s, application of biotinylation labelling of the entire circulating blood cell population within animal models not only provided the first proof that reticulated

platelets are indeed newly formed platelets but are more reactive than mature platelets ***(Robinson et al., 2000)***. Immature platelet fraction (IPF) is the percentage of reticulated platelet (RP) of total platelet count ***(Cho et al., 2007).***

Flowcytometric detection of reticulated platelets:

Clinical laboratory testing for platelet function includes the bleeding time and platelet aggregation, both of which are somewhat limited in their ability to predict platelet dysfunction or hyperfunction. Research studies have employed plasma or serum measurement of glycocalicin, platelet factor 4,β - thromboglobulin, and other secreted products to study platelet function and activation in a variety of disease settings; however, these assays may be influenced by normal metabolism and excretion of the measured analytes, making interpretation difficult ***(Lakkis et al., 2004)***.

Direct examination of the platelet's state of activation, as well as the platelet's ability to undergo secretion and activation to agonist, is a highly desirable method for examining platelet function. Many investigators have turned to flowcytometry to provide a method for platelet analysis in whole blood or in minimally purified platelet preparations ***(Rajantie et al., 2004)***.

A. General Methodologies:

Platelets are visualized by flowcytometry identically to

methods used for leukocytes and other cell populations of interest. Because of their relatively high concentration, platelets can be rapidly examined by flow in very small blood volumes. Flow methods allow for identification of platelet subsets based on antigen expression at rest or after stimulation with platelet agonists ***(Guo et al., 2003)***.

The principles of flowcytometric examination of platelets are similar to other cells. Platelets in a sample are incubated with fluorescent probes, then placed on the flowcytometer. Suspended platelets are drawn into the flow chamber and through the beam of a laser; activation of the fluorophore occurs, and the emitted fluorescence and light scatter properties of the platelets are detected. Because of the platelet's small size and unique receptor properties, the photomultiplier detectors on the flowcytometer must be set specifically for platelet fluorescence and light scatter. In general, platelets cannot be adequately examined using leukocyte settings ***(Nakamura et al., 2002)***.

Flowcytometric analysis of platelets in whole blood can be performed with extremely low blood volumes (as low as 5μL), easily allowing platelet testing in neonates and infants. For example, ***Rinder et al., (1994)*** examined platelet activation using monoclonal antibodies (MoAb) to the platelet α-granule antigen, CD62 P, and the surface membrane receptor, glycoprotein (GP) Ib/IX, in pediatric patients with congenital heart disease (CHD) undergoing cardiopulmonary

bypass (CPB). CD62 P is a neo antigen expressed on the platelet surface only after α-granule release and thus serves as a marker for this aspect of platelet activation; GPIb/IX is a complex on the platelet surface which is partially lost from the platelet surface with activation, through cleavage and internalization ***(Peterec et al., 1997)***.

Platelet turnover can also be examined in neonates by detecting the percentage of younger, reticulated platelets (RP) in very small amounts of whole blood or platelet-rich plasma ***(Peterec et al., 1997)***.

B. Detection:

Examination of the RNA content of circulating platelets using the nucleic acid-binding dye, thiazole orange (TO), and flowcytometry has disclosed a subset of high RNA-containing platelets, reticulated platelets (RP) ***(Kienast and Schmitz, 1990; Ault et al., 1992; Rinder et al., 1993)*** . The percentage of circulating RP, as compared with the total platelet population, was shown to be increased in patients with peripheral platelet destruction, suggesting that RP represent those platelets which have been most recently released from the marrow into the circulation ***(Rajantie et al., 2004)***. Therefore, in thrombo-cytopenic individuals due to peripheral platelet destruction who have a normal marrow response, the RP value should indicate platelet turnover ***(Chaoui et al., 2005).***