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Functional Status of Platelets and Hereditary Platelet disorders

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ABSTRACT The circulating platelets are derived from bone marrow megakaryocytes in response to thrombopoitic growth factors. The clinical response with recombinant human thrombopoietin (Peg-VHUMGDF and Vh TPO) is being evaluated in chemotherapy induced thrombo-cytopenia. The pathophysiology of platelet adhesiveness in flowing blood is a complex process that involves fibronectin, collagen, platelet glycoproteins thrombo-spondin and a host of related components. Various inherited platelet disorders involve glycoproteins, Von Willebrand factor and release from platelet storage granules leading to von Willebrand disorder (vWD) thrombasthenia, Bernard Soulier disorder, macrothrombocytopenia, storage pool disorder and collagen receptor defect. The constituents released from adherent platelets modify the vessel tone vessel wall repair and coagulation.

INTRODUCTION

The blood platelet is derived from megakaryocytes but the process of platelet production – thrombopoisis remains still a subject of wide speculation. Megakaryocytes are large, polyploid bone marrow cells . In recent years *in vitro* cloning of progenitor cells and monoclonal antibodies has advanced our knowledge of megakaryopoisis and the effect of thrombopoisis factors in the understanding of physiology of production of circulating platelets. Megakaryocytes send pseudopodia, a highly redundant demarcating membrane, out into bone marrow sinusoids and platelets are budded off. In experimental model with mice recovering from severe thombocytopenia, there are increase of Protoplatelet – platelets in the bone marrow sinusoid, whereas the mice lacking the transcription factor NF-E2 or GATAI shows persistence of thrombocyto-penia.

Platelet count of an individual is fairly constant throughout but there is large variations ranging from 150-450 x 10^9. There is an inverse relationship between the normal platelet count and normal mean platelet vol. of 4.7 fl. The bone marrow megakaryocytes respond to demand of platelets by increasing their number, size and ploidy .

Thrombopoitin Growth Factors – Platelets are fundamental to hemostasis in the body. In recent years, attempts are made to understand the extracellular growth factors that are important in regulating the process. A number of growth factors have been identified which may stimulate megakaryocytes – platelet formation. Hemopoietic growth factors and its effect of megakaryocyte came from studies on recombinant granulocyte macrophage colony stimulating factors (G-CSE) and interleukin-3 (IL-3). However, the effect has not been translated to significant clinical benefit. IL-6 family of molecules had more clinical benefit to thrombopoisis. These molecules need signal transducing receptor molecules gpl 30. IL-11 which reduces the need for platelet trans-fusion in specialized clinical setting [17,19, 30].

IL-11 identified as a stromal cell – derived growth factor, acts on multiple hemopoietic lineage. Megakaryocytes express L-11 receptors thereby producing evidence for direct effect of IL-11. However, IL-11 can augment the megakaryopoitic activity of other cytokins e.g. IK-3, SCE, TPO (thrombopoitin). IL-11 *in vivo* enhances megakaryocyte progenator, consequently enhances platelet production. However, there is possibility of TPO might mediate thrombopoisis in responase to other growth factor including IL-11, IL-6 and SCF [16, 22, 31].

TPO, a principal platelet regulator, consists of a receptor binding domain and a carbohydrate rich carboxyl terminus which is important in protein stability[8]. TPO increases the size and number of megakaryocytes and stimulates the expression of platelet specific markers. The evidence suggests the role of TPO signaling early thrombopoisis. Although clearly beneficial, IL-11 is associated with significant

adverse effect [18, 24]. The clinical efficacy of two forms of TPO, pegylated recombinant human megakaryocyte growth and develop-ment factor (PEG-rHUM GDP) and rTPO. However, the mode of administration of rTPO (I.V. route) and rHU GDF (S.C. route) is associated with antibody production and thereby limiting the efficacy [38, 41].

The predominant sites of TPO production are liver and to lesser extent, the kidney and smooth muscle, Serum TPO level is increased in chemotherapy induced thrombocytopenia, chronic thrombocytopenia, aplastic anaemia and leukaemia. In a recent successful clinical trial with TPO in a severe chronic thrombo-cytopenia case in a pediatric age group, resistant to any treatment, revealed that bone marrow cells responded consistent with those of age matched children without any plasma inhibitor [42]. However, individual evaluation of clinical status is required before clinical trial of TPO in chronic thrombocytopenia.

In phase I/11 clinical trials in cancer patients receiving myelosuppressive chemotherapy demonstrate clinical safety and platelet stimulating activity. Treatment with a single dose of rTPO resulted dose dependent 62.1% to 212% increased in circulating platelet count. The platelets appear morphologically and functionally normal [21]. No patient in the trial developed antibodies to PEG-rHUM GDF [40].

Utilisation of Circulatory Platelets – In healthy state, the rate of platelet release from megakaryocytes is equivalent to rate of utilization in the circulation[7]. Platelets circulate for a variable period ranging from 2-9 days in thrombocytopenic subjects transfused with large volume of platelets [40]. However, in healthy recipient life span may be for 5-10 days [40]. The life span of blood platelets as determined by labeling platelets with radioisotope present much conflicting results [8, 9]. The apparent difference in the kinetics of circulating platelet could be due to selective labeling of platelet, apart from reutilization or elution of label [6]. Extrapolation of the platelet utilization rate of circulating platelets from observed survival of labeled platelets may not uniformly reflect the coagulation status in clinical or experimental study.

In the absence of a suitable standard of life span study,unlike that of red blood cells, platelets simultaneous labeling with two different isotopes, one for random and other for cohort platelet and clearance rate provide a more reasonable model[8]. Haemorrhagic manifestation on myloproliferative disorder (MPD) have been attributed to functional deficiency of platelet. However, the survival of isotopically labeled platelets *in vivo* in PMD has been reported as increased, decreased or even normal [7]. This apparent variations of the survival of platelets could be due to random loss of cytoplasmic constituents to which one or other isotope has been incorporated [6].

Platelet Structure

The circulating platelets are fragments of megakaryocytes, 1-3 μm in diameter with average volume of 4-7fl. The detailed structure as revealed by electron microscope shows a plasma membrane which is a limiting membrane, a submembrane which is a link between plasma membrane and inner cell structure, a cytoskeletal and organelle consisting of granules, dense bodies, lysosomes and mitochondria[16]. Alfa granules are spherical bodies which release coagulation factors, platelet derived growth factors (PDGF) and thrombospondin involved in platelet aggregation, platelet factor 4 (PF4) and B-thromboglobulin. PF4 binds endogenously released heparin. Alfa granules also contain fibrinogen and factor five (FV), which are released after platelet activation. However, various proteins are absorbed from plasma by platelets and megakaryocytes. Alfa granules also release PDGF but it is not specific to platelet alone. Dense bodies measuring 250-350 μm indiameter contain ADP, ATP, GTP, Calcium, Magnesium and Serotonin [19, 21]. ADP is the important component secreted from the dense bodies after platelet activation and when released ADP binds to specific receptors to initiate platelet aggregation. However, ADP in rapidly degraded to adenosine which inhibit further platelet activities by enhancing cyclic AMP levels. The calcium concentration of microtubulary activating membrane phospho-lipidases enhances liberation of arachidonic acid required for platelet function.[11-13]

Platelets are known to contain high concentration of L-ascorbic acid and a relationship between ascorbic acid and platelet functions like aggregation and adhesiveness has

been reported [12]. Further studies on leukaemic platelets showed that some of the enzymes (dehydroascorbatase, xyliton dehydrogenase) are reduced or completely absent [3]. Lysosomes are vescicles containing a number of acid hydrolases including β-glucoronidase, peroxidase, elastase, heparinase and enzymes that degrade polysaccharidase. Platelet lysosomal contents are slowly released and digest materials that platelet endocylose but platelets have limited ability to phagocytose.

Intracellular distribution of acid hydrolases in normal and leukaemic platelets show some subtle differences [34]. However, other lysosomal enzymes e.g. alkaline phosphatase activity of leukaemic platelets is not changed[18] but increase of acid phosphatase, B-Glucoronidase and decrease of succinate dehydrogenase activity are observed in leukaemic platelets.[36] The mechanism of controlling system of alkaline phosphatase activity differs in leukocytes and platelets[10, 26]. This difference could be due to the alteration in structural or regulator genes of Ph_1 chromosomes of leukaemic leucocytes or to the difference of the control system of the enzyme activity at cytoplasmic level, although megakaryocytes and myloblast may originate from common stem cells. If a definite control system exists in the precursor cells with respect to their cellular kinetes it is presumed that the changes in enzyme activity of blood cells will follow wherever there is any alteration of cell population involving any or more cell. Intracellular granules of platelet also store, apart from hydrolases, scrotonin (5-HT) which is released during aggregation and acts as an agonist and promotes further aggregation and alter membrane fluidity and permeability [27]. Cardiovascular disease including hypertension or vasospasm may have a link to the exaggerated liberation of serotonin from aggregating platelets [26].

5-HT is accumulated by platelets in multi-step process which involves transport across the platelet membrane via a mobile carrier and storage in intracellular granules.Various agonist-induced aggregation and release require an elevation of intracellular calcium (Fig. 1).

Platelet factor 4 (PF4) a constituent of alfa granules, binds endogenously released heparin and neutralizes heparin; stability to inhibit coagulation. PF4 binds to heparin on the endothelial wall and can, therefore, delay thrombin neutralization after platelet release.The normal content of PF4 in unstimulated platelets is about 18 mg/10^9. Anticoagulant, even in small subcutaneous dose prevents post-operative deep vein thrombosis and large intravenous dose inhibits intravascular coagulant [10]. Platelet heparin action is somewhat paradoxical since small dose reduces platelet adhesiveness and large dose enhances platelet sequestration. There is apparent high rate of utilization of circulating platelets even on the state of hypocoagulation with large dose of heparin [28]. The action of collagen and ADP on platelets is associated with sharp change, aggregation and release reaction which occur in parallel with formation of 1,2-diacyl-glyceric phospholidic acid, arachidonic and metabolites. Collagen stimulation of blood platelets results in significant increase of malondialdehyde (MDA). The vascular endothelial production of platelet inhibiting substances – Prostacyclin was found to be depressed with a simultaneous elevation in serum thromboxane B2 levels indicating increased formation of thromboxane A2 in platelets.[27]

Heavy metals like mercury and cadmium are known to cause lipid peroxidation by generation of free radicals and perturbation of the antioxidant protection mechanism of cells. Interaction of human platelets *in vitro* were studied with respect to the platelet activation process as indicated by melandialdehyde formation and also to the components of the cellular antioxidant defence system such as catalase, glutathione peroxidase (GP), glutathione reductase and reduced glutathione (GSH). The cadmium effect on platelet enhances the formation of MDA via the cyclooxygenase pathways involving intraplatelet accumulation of cadmium which is inhibited by calcium.[29] Cadmium is also shown to increase the responsiveness of platelets to aggregating agent.[14] Serotonin (5-HT) is released during platelet aggregation which itself acts as an agonist and promotes further aggregation.[36] 5-HT is accumulated by platelets in a multisep process which involves transport across the platelet membrane via mobile carrier and storage in intracellular granules. The release of platelet serotonin over a period corresponds to the platelet life span[20].

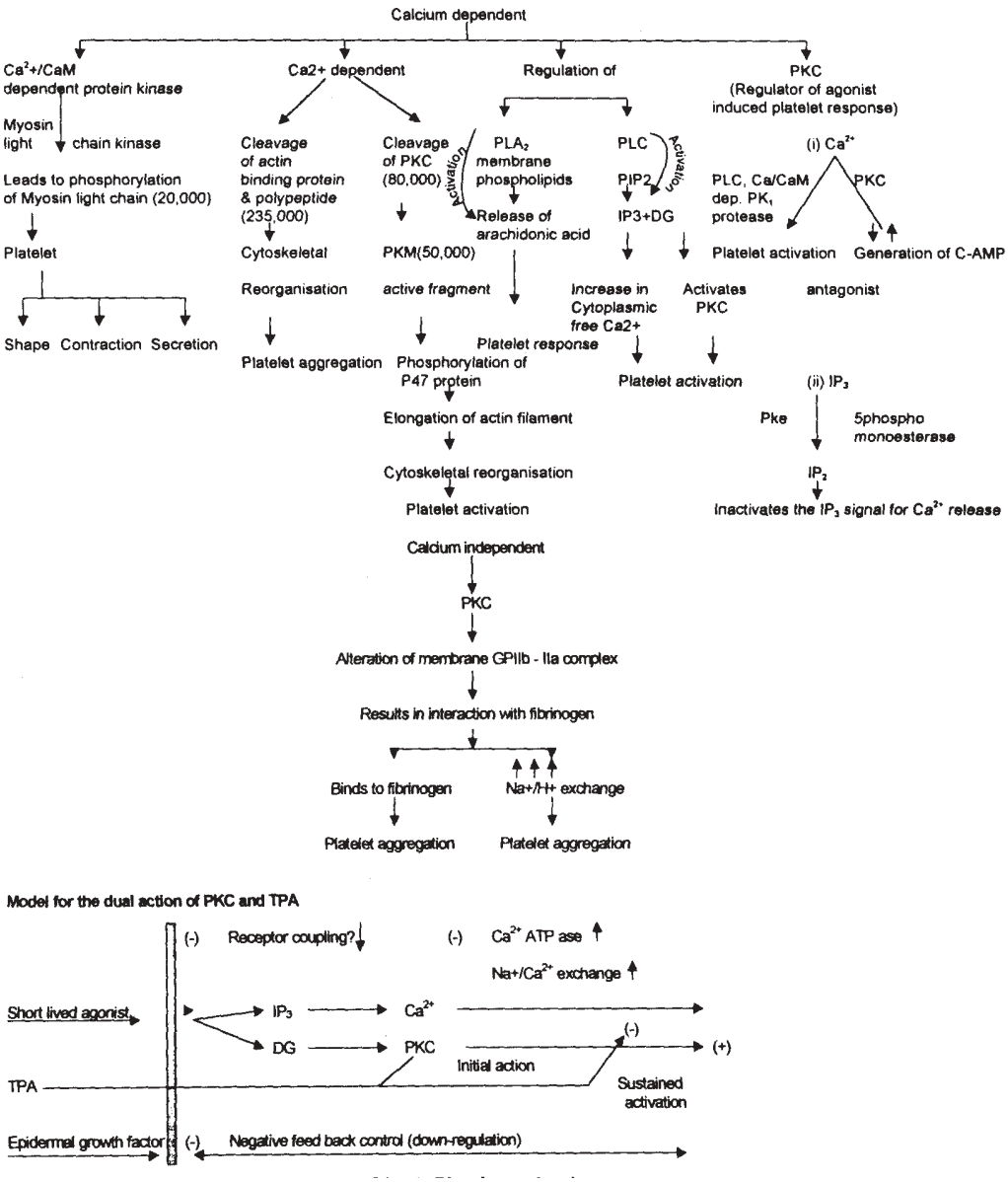

Fig. 1. Platelet activation process

The Phospholipid-Inositol Complex has been shown to be important to cell signaling. The impaired formation of this lipid complex in response to growth factor (platelet derived growth factor – PGDF) decreases mitosis in the cells so impaired. 3-Phosphorylated phospha-tidylinosilides (3-PPI) has a role in platelet function since it leads to failure of platelet to form 3-PPI in response to stimulus and a corres-ponding abnormal morphological event.[4]

Platelet Adhesion and Aggregation: Platelet adhesion occurs in flowing blood and a special perfusion system has been developed to mimic

such flow condition. However, it is the wall shear rate and not the flow rate that is essential for adhesion [4]. The importance of other physical factors, apart from wall shear rate, e.g. haematocrit, red cell deformability, plasma viscosity and pulsability need to be considered.

In the vessel wall collagen von Willebrand factor (vWF) and fibrinectin are important adhesive proteins. Fibrinogen infiltrates the vessel wall caused by tissue factor-dependent coagulation and platelets adhere to fibrin. Lamilin, present in basement membrane in association with Collagen IV and Thrombospondin present in the matrix of vessel wall are important determinant of platelet adhesion to vascular wall. Minor injury to vessel wall (desquamation) leads to adhesion of platelets to subendothelial layer and activates collagen, thrombospondin, laminin, vitronectin and tissue factors hitherto remaining inactive. Platelets do not release their constituent at this stage nor promote platelet aggregation. More severely injured vessels expose deeper structure of vessels to platelet and not only induce platelet adhesion but also facilitate platelet aggregation by activating release reaction.

This difference in platelet response are attributed to composition of vessel wall. Collagen I and Collagen III are synthesized by smooth muscle cells at deeper layers and promotes platelet aggregation, whereas Collagen IV and V which are synthesized by endothelial cell promote platelet adhesion. Platelet adhesion to Collagen requires plasma protein (vWF) which acts on platelet glycoprotein receptor complex (GP1b/1x) followed by activation of GPII b/IIIa which are synthesized and located below the endothelial lining and promotes platelet adhesion. Platelet adhesiveness is potentially inhibited by 13-hydroxyoctade-cadienoid acid (13-HODE) possibly by inhibiting Thromboxane A_2 by endothelial cells, released into tissue immediately underneath and inhibit platelet adhesion to surface table 1.

A number of active agents e.g. thrombin, epinephrine, TxA_2, arachidonic acid metabolites cause platelets to release their granular contents and activate platelet aggregation and coagulation system.

Platelet aggregation is induced by a variety of stimuli, e.g. ADP, TxA_2, thrombin, collagen and epinephrine. The platelet aggregation is preceded by changes in the platelet morphology particularly microtubular system and microfilament.

Table 1: Some hereditary disorders of platelets

S. No.	*Disorders*	*Major locus of defects*
1.	von Willebrand disorder (pseudo)	Glycoprotein 1b, la/11a, IV
2.	Glanzman thrombasthenia	Glycoprotein 11b/IIIa
3.	Bernard Soulier Syndrome	Glycoprotein 1b/V
4.	Wiskott-Aldrich syndrome	Membrane cytoskeleton abnormality
5.	Storage pool disorder	Abnormality of platelet granules
6.	Gray platelet syndrome	Alfa storage deficiency

ADP is released from dense granules of platelet in response to collagen, epinephrine or TxA_2. The action of ADP depends on specific platelet glycoprotein receptor known as GPIIb/IIIa. Thrombin induced platelet aggregation by releasing ADP which induces secondary irreversible platelet aggregation. Thrombin also activates the platelet membrane phospholipases initiating formation of TxA_2 – an arachidonic acid metabolite which induces platelet aggregation. The free arachidonic acid liberated from activated platelets by any of the agents mentioned, from platelet phospholipid, is oxidized to Endoperoxidase PGG_2 and PGI_2 catalyzed by the enzyme Cyclooxygenase. This step is important since it is inhibited irreversibly by aspirin. Arachidonic metabolism is affected in Myeloproliferative disorders and explains their bleeding tendency [33]. TxA_2 promotes platelet aggregation directlyand also synergistically with ADP.

The main role of platelet agonist is to activate receptors for fibrinogen on the platelet membrane. The platelet receptor recognition site occurs in the variant of the γ chain of fibrinogen. Studies of platelet aggregation is essential in a suspected platelet functional defect, particularly in patients with borderline platelet defect. The standard method utilizes an optical aggregometer essentially based on turbidometric method [13]. There are many platelet aggregating agents that activate platelet aggregation [15]. ADP binds to platelet membrane receptor associated with the glycoprotein GPIIb/GPIIIa and extracellular calcium ions. Fibrinogen then binds in a

reversible fashion to the activated membrane complex. After initial activation by ADP, the platelet undergoes a change in shape and induces initial reversible aggregation by bound fibrinogen, which aids cell to cell contact – a primary aggregation response. This primary aggregation due to low dose ADP is reversible but with higher concentration of ADP causes an irreversible secondary wave of aggregation that is associated with alfa and dense granule release. ADP stimulates release of arachidonic acid and TxA_2 generation as well as inhibits adenylate cyclase activity. The secondary irreversible wave of platelet aggregation in response to ADP is abnormal in certain states (Fig.1). Aspirin and non-steroidal anti-inflammatory agents, alcohol and some abnormalities e.g., Cyclooxygenase deficiency, Thromboxane A_2 receptor abnormally or deficiency of platelet granules or release of contents. In storage pool disease in which alfa or dense granules or both are deficient.

Aggregation to collagen in platelet aggregometry is preceded by a short 'lag phase' lasting for about a few seconds. The duration of the length of lag phase in response to a given dose of collagen is noted for platelet response to collagen. The lag phase is succeeded by a single wave of platelet aggregation. This activation of platelets by collagen is via the platelet prostaglandin pathway and formation of TxA_2 from membrane phospholipid. However, the mechanism is dose dependent of collagen in the reaction. Ristocetin interacts with FVIII related antigen VIII RAg von Willebrand factor (vWF) and glycoprotein GP1b on the platelet surface inducing platelet aggregation. Defective aggregation to ristocetin is observed in vWF disorder or Bernard Soulier Syndrome. Calcium is probably the single-most important mediation in platelet activation.

INHERITED DISORDERS AFFECTING PLATELET ADHESION AND AGGREGATION

von Willebrand Disease (vWD) is due to absence or abnormality of vWF. The absence of vWF leads toType I and III causing a defect in platelet adhesion at high shear rates. The platelet adhesiveness is inversely proportional to collagen activity of the vascular wall, because vWF releases from platelet alpha granules may participate in adhesion. In vWF Type 11a shows decrease in interaction with platelets in the ristocetin cofactor assay due to low molecular weight. In Type 11b vWD shows increased interaction of vWF with GP1b on platelet – determined by increased aggregation of platelets by low concentration of ristocetin.vWF is synthesized by the endothelial cells is not abnormal in platelet adhesion, but abnormality appears when this vWF circulates. The mechanism of action of vWF has been postulated as Type 11b. vWF coats platelets on GP1b, thereby acting as a competitive inhibitor of the function of GP1b (Table 1).

Bernard-Soulier Syndrome is a form of macrothrombocytopenia characterized by absence of or abnormality of GP1b/1X Complex. In such condition the platelets shows abnormality of adhesion in all situations where vWF/GPIb interaction is necessary for platelet adhesion.

Storage Pool deficiency shows reduction of storage pool of ADP, ATP and serotonin due to reduction or absence of dense granules. The platelet adhesion is not affected out aggregation formation is reduced [42].

Collagen receptor deficiency is an extremely rare condition where the patient shows a defect in platelet adhesion to the vessel wall and to subendothelium and a few platelets that adhere

Table 2: Different variants of vWF Disorders

Test profile	*Types*			
	I	*IIa*	*IIb*	*III*
Bleeding time	Prolonged/Normal	Prolonged	Prolonged	Prolonged
FVIIIC Ag	Decreased	Decreased/Normal	Normal/decreased	Decreased
Ristocetin induced aggregate	Normal/reduced	Reduced	Increased	Reduced
vWF: Ag	Decreased	Normal/Decreased	Decreased/Normal	Decreased/absent
VWF: Ristocetin Cofactor	Decreased	Decreased	Decreased	Decreased/absent
Plasma multimers	Normal/reduced	Absent/normal	Normal/absent	Variable

Table 3: Laboratory diagnosis of some inherited platelet disorder

		Platelet	*Functional*	*Status*	
Parameters	*Storage Pool Defect*	*Bernard Soulier*	*Glanzman*	*Afibrinogenemia*	*Von Wille-Brand*
Platelet Count	Normal	Normal/decrease	Normal	Normal	Normal/decrease
Bleeding time	Prolonged	Prolonged	Prolonged	Prolonged	Prolonged
APTT	Normal	Normal	Normal	Increased	Normal/Increased
Fibrinogen	Normal	Normal	Normal	Decreased	Normal
ADP response	Absent	Normal	Absent	Absent	Normal
Ristocelin response	Normal	Absent	Normal	Normal	Absent

show a defect in spreading [32].

Macrothrombocytopenia in characterized by low platelet count with large platelet size. The total platelet volume is not reduced and comparison of platelet adhesion with that of normal platelet of similar platelet counts shows no platelet adhesion defect [37]. The salient features of the hereditary platelet disorders and their variations are given in Table 3.

CONCLUSION

The blood platelet should be present in circulating blood in adequate number and with optimum functional status. Platelet functions can be evaluated only when platelet number is adequate and usual tests are skin bleeding time, platelet function (PF3) assay and platelet aggregation study against agonist. Platelet aggregometry can pin point some acquired as well as inherited platelet disorders (Table 3) in relation to ADP, epinephrine, ristocelin, collagen, thrombin and arachidonic acid. The ristocelin co-factor activity and VWF : Ag assay can diagnose von Willebrand disorders including its subtypes.

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