Home

THROMBOPOIESIS AND THROMBOKINETICS: AN APPROACH TO THE EVALUATION OF THROMBOCYTOPENIA

MEDICAL GRAND ROUNDS

UNIVERSITY OF TEXAS HEALTH SCIENCE CENTER AT DALLAS

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RICHARD G. SHEEHAN, M.D.

THE MEGAKARYOCYTE

The study of thrombopoiesis has lagged behind the characterization of erythropoiesis and granulopoiesis because of two primary factors: (a) the cell recognizable by light microscopy as a megakaryocyte (MK) represents only a minor fraction of bone marrow cells (in the range of 4.4 per 10^4 bone marrow nucleated cells, i.e., 0.04 percent) and therefore cannot be accurately quantitated by simple techniques and (b) in vitro colony growth of MK has been, until recently, non-measurable due to the fact that MK colonies are very small in number and contain very few cells and went unrecognized in cell cloning systems. The development of additional methods for identifying MK has led to a breakthrough in enumerating their presence and studying their in vitro growth patterns and control. (1) An additional problem, of less certain significance, in evaluating human megakaryopoiesis and thrombopoiesis, has been the fact that most studies have been carried out in rodent systems and the question of species variation in growth patterns and control mechanisms has not yet been answered.

The light microscopy recognizable MK population has been divided into four arbitrary stages of maturation based on nuclear and cytoplasmic characteristics. (2,3) These are generally very large cells compared to other BM myeloid lines and they possess the unique feature of being hyperdiploid. All MK recognizable by light microscopy have the minimum of 4N (tetraploid) chromosome complement, the 8-16N population representing the most frequent cells. Properties of these stages of maturation are shown in Table 1.

Cytologic characteristics of megakaryocyte maturation stages. In the first column are diagrams of the nuclear configurations and nuclear:cytoplasmic ratios at each stage. Much of the size variation for each stage is related to differences in ploidy level (Levine *et al.* 1982). The likely ploidy levels of each stage are suggested in Fig 1.

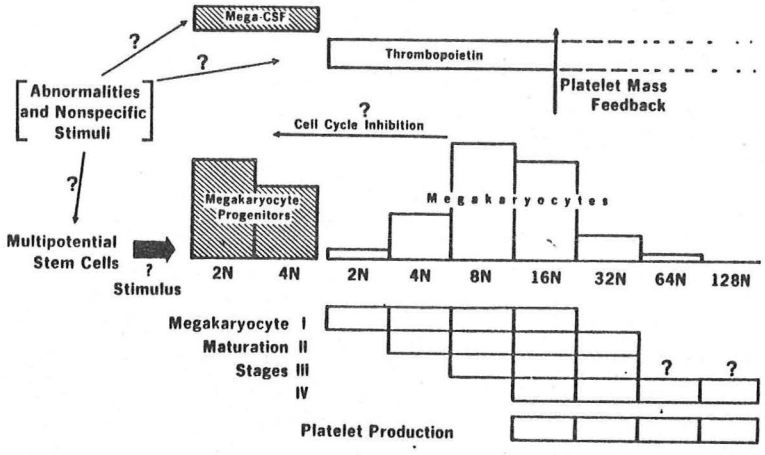
	Stage	Nuclear morphology	Cytoplasmic staining (Wright-Giemsa)	Approximate size range	Demarcation membranes	Granules	Suggested name
	I	Compact (lobed)	Basophilic	6–24 μm	Present by electron microscopy	Few present by electron microscopy	Megakaryoblast
	II	Horseshoe	Pink centre	14–30 μm	Proliferating to centre of cell	Starting to increase	Promegakaryocyte
	III	Multilobed	Increasingly more pink than blue	16–56 μm	Extensive but assymetric	Great numbers	Granular megakaryocyte
	IV	Compact but highly lobulated	Wholly eosinophilic	20–50 μm	Evenly distributed	Organized into 'platelet fields'	Mature megakaryocyte

Table 1. Reference 2

Essentially all of the surface antigens and cytoplasmic and granule content of the anucleate platelet are acquired prior to the final demarcation and release of individual platelets by a MK. Many are acquired very early in MK development. (4,5) The production of heterologous or monoclonal antibodies to many of these membrane or cytoplasmic substances has allowed for the use of immunofluorence methods of identification not only of light microscopy recognizable MK but also earlier stages of maturation and may allow for some degree of selectivity in assessing stages of differentiation. Use of these markers has led to the identification of a greater number of MK committed cells in bone marrow, accounting for perhaps another one-third of the total, presently designatable, MK population. (6,8)

MEGAKARYOCYTE DEVELOPMENT

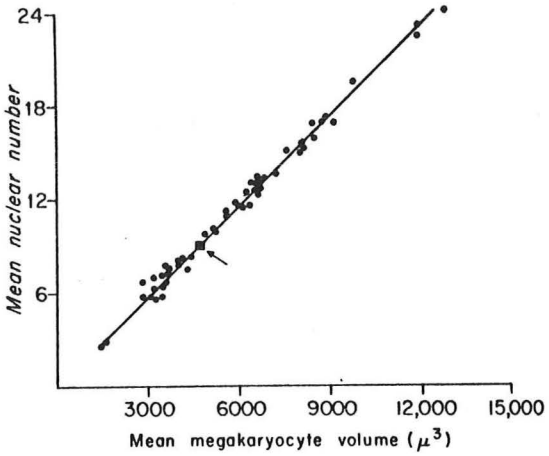
Two primary processes are responsible for the eventual development of the mature platelet producing MK. (Fig. 1) Initially, proliferation of MK precursors occurs, presumably after commitment from the pluripotent hematopoietic stem cell pool. (2) This process provides the primary mecahnism for amplification of MK numbers by the usual mechanisms of cell division. Under normal steady state conditions, only a small fraction, perhaps 20% or less, of MK precursors are in cell cycle. (9-11) At some point in time, the proliferating MK precursors undergo the second stage of commitment, that is to actual maturation and differentiation into platelet producing MK. (2) There is evidence in rodent systems that more than one population of MK precursors exist based on the relative propensity to undergo continual proliferation as opposed to commitment to maturation and cessation of cell (though not nuclear) division. (12) Whether selective stimulation



Megakaryocytopoiesis. Shown horizontally in the centre is the progression from uncommitted multipotential stem cells, through the proliferating precursors detected in the *in vitro* cloning assays, to the spectrum of maturing megakaryocytes. The relative DNA levels of megakaryocytes and their precursors is given as ploidy values (N) where 2N is a diploid cell. The megakaryocytes are also represented vertically in terms of their maturation stages, ending in platelet shedding; a detailed classification of these stages is given in Table I. The columns show the maturation stages at particular ploidy ranges. Thus, 4N megakaryocytes are found at maturation stages I and II. The top row shows a postulated two-level regulatory process with megakaryocyte colony stimulating factor (Mega-CSF) primarily influencing proliferation of the clonable precursors and thrombopoietin required for megakaryocyte ploidy amplification and possibly for maturation. It is not certain that these regulators are completely exclusive in their target cell specificities as shown. Furthermore, no clear distinction is currently possible between the specific or nonspecific control of the two factors. Thrombopoietin production is sensitive to variations in platelet mass, as indicated in the figure; it is assumed that this feedback mechanism operates on the source of thrombopoietin. The progenitors might be controlled by cell cycle inhibition, perhaps as a consequence of normal numbers of megakaryocytes.

Figure 1, Reference 2

or suppression of one or more of these populations occurs in pathologic states remains to be determined. The maturation process is mechanistically unique to MK among BM cells. Further cell division ceases and the increase in platelet producing capacity is accompanied by continuing mitoses without cytokinesis, i.e. endoreduplication resulting in increasingly higher degrees of hyperdiploidization (ploidization). (13-16) In general, the average MK of a given ploidy number is larger, with more cytoplasmic content, than those of less ploidy and appear to produce more platelets. (3,17,18). Fig. 2. The 8N-16N MK predominate in normal BM with few if any MK beyond 32N except in pathological states. (15,16) The absolute number of platelets produced per unit time, therefore, appears to be both a function of the absolute number of mature MK as well as the mean ploidy and volume for that population. Harker has termed the combination of a MK nuclear lobe and its associated cytoplasmic material a nuclear unit and the number of platelets produced by a nuclear unit appears to be rather fixed. (18) MK maturation is further characterized by the progressive production of cytoplasmic organelles, i.e. the granule and microtubule content of platelets as well as the development of a demarcation membrane system, which, at maximum maturity, is responsible for the final step of individual platelet isolation and release. (14,19)



A straight-line relationship between the average number of nuclei and the volume of the megakaryocyte indicated that these determinations are equivalent measurements. The normal value ±1 SD is located by the arrow.

Figure 2, Reference 18

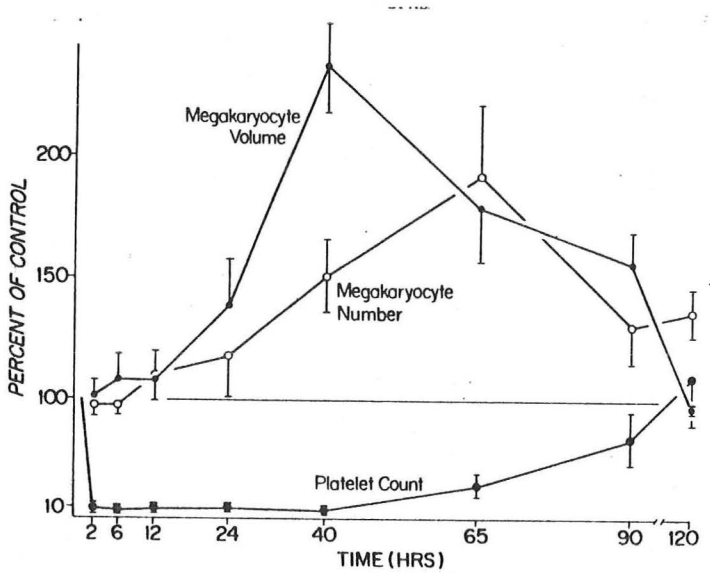
CONTROL OF MEGAKARYOPOIESIS AND THROMBOPOIESIS

As indicated, the bulk of data on control mechanisms of platelet production has been accrued from the study of rodent systems. (1) The first most important contributions to the understanding of human platelet production were pioneered by Harker utilizing an imaginative, though tedious, technique of measurements of thrombokinetics and megakaryocyte characteristics. (18) Most of his observations, however, have to date withstood the test

of time. Steady state thrombopoiesis and responses to physiologic and pathologic perturbations appears to be mediated at more than one stage of the previously described steps in MK proliferation and maturation (20).

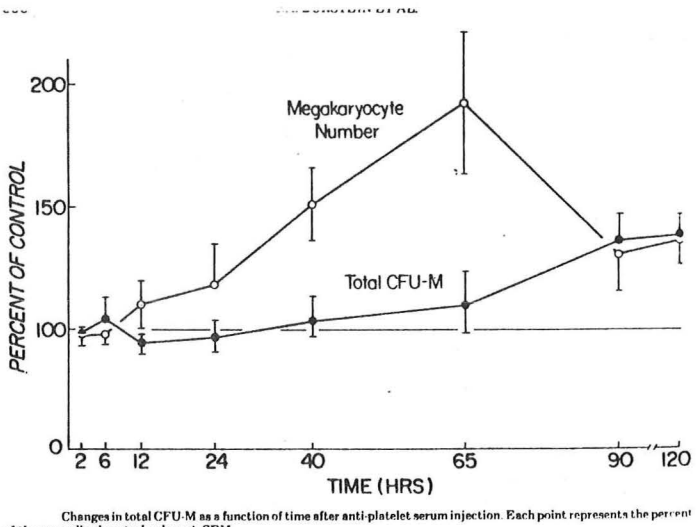
THROMBOPOIETIN (TPO) or THROMBOPOIESIS STIMULATING FACTORS (TSF)

As an analogy to the extensively studied humoral stimulator of erythropoiesis, attempts have been made to identify a similar thrombopoiesis stimulating factor(s) (21,27). Problems encountered have generally been related to the development of a truly physiologic and reproducible assay for such activity as well as a major, and as yet unsolved, inability to isolate and purify such substances. Methods employed have utilized both experimentally induced thrombocytopenia in animals (7,10,22-30) and thrombocytopenic states in man (18,31,32) to evaluate perturbations of normal megakaryopoiesis and thrombopoiesis. These have included morphologic and kinetic studies of MK and platelet production in such settings as well as the effects of injection of plasma from thrombocytopenic subjects into experimental animals (32). More recently, the ability to establish in vitro colony growth from both rodent and human bone marrow has allowed the study of the effects of such perturbations on MK precursor proliferation (10,23,26,29,30)(Fig. 3,4). It is presently believed that one or more stimulators of thrombopoiesis are produced acutely in response to the experimentally induced thrombocytopenia in animals. These stimulators have been termed thrombopoietin (TPO) or thrombopoiesis stimulating factors (TSF). The standard assay is a crude bioassay evaluating the incorporation



Changes in platelet count and megakaryocyte number and volume as a function of time after a single injection of anti-platelet serum. Each point represents the percentage of the normalized control values.

Figure 3, Reference 10



Changes in total CFU-M as a function of time after anti-platelet serum injection. Each point represents the percent of the normalized control values ± SEM.

Figure 4, Reference 10

of radiolabelled tracers into MK and the subsequently released platelets after producing thrombocytopenia or after injecting serum from thrombocytopenic animals or humans into normal recipient animals. (24) The general physiologic effects of these manipulations are summarized in Table 2. It is of interest

OBSERVED EFFECTS OF THROMBOPOIESIS

STIMULATING FACTORS

Incorporation of Isotopic Labels into MK and Platelets

Increased Number Immature MK

Increased MK Ploidy and volume

Increased Mean Platelet Volume

No Increase Mega-CFU in vivo

No Stimulus to Mega-CFU Growth in vitro

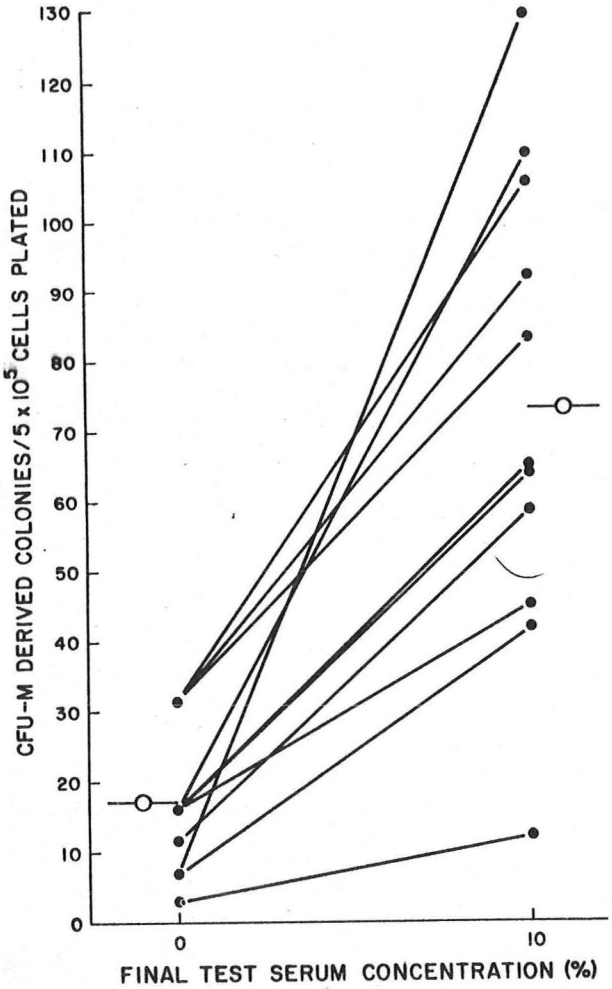
Table 2

that, in most studies, the injection of TSF does not result in an increase in the circulating platelet count. These characteristics are of interest since they reproduce many of the findings seen in human bone marrow of patients suffering from peripheral destructive thrombocytopenia. (18).

It is presently accepted that TSF(s) do exist and that their primary function is to stimulate MK maturation and perhaps commitment from the proliferative pool into the differentiation pool. The mediator of TSF production/secretion is felt to be the circulating platelet mass since TSF activity is found in serum of thrombocytopenic subjects and experimentally induced or clinical thrombocytosis results in a reduction in immature MK and a decrease in MK ploidy and mean platelet volume. (27).

MEGAKARYOCYTE COLONY STIMULATING ACTIVITY (MK-CSA)

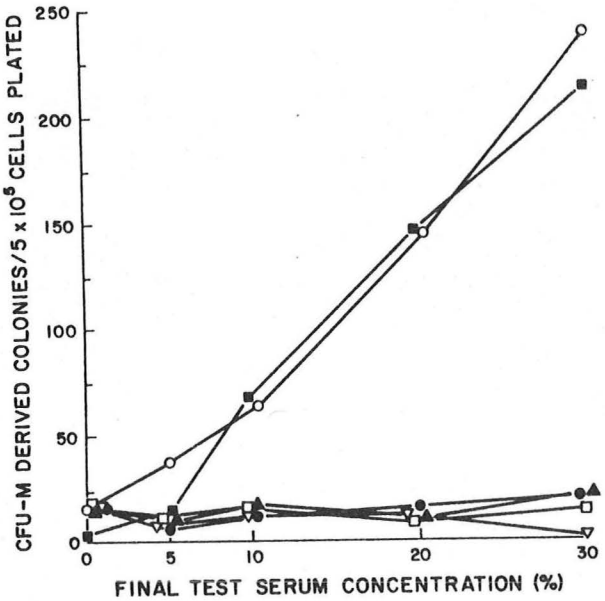
A significant advance occurred in the study of thrombopoiesis with the development of in vitro culture systems for MK colony formation. (1). As noted, relative to erythroid and granulocyte-macrophage colony forming activity, the BM contains few MK colony forming units (MK-CFU) and each colony has a small number of cells. Their identification became possible with the use of fluorescent tagged antibodies to specific MK-platelet antigens. (5-8). Optimal in vitro colony growth is dependent upon two types of factors: (a) MK colony stimulating activity (MK-CSA) which can be defined as an obligatory substance necessary for the stimulation of MK colony formation, and (b) potentiating factors which allow for optimal number and size of colonies formed. (1,20,30,33-39)(Fig. 1). The latter factors appear to have the characteristics of TSF, i.e., promote MK differentiate and therefore allow recognition of MK colonies. (1,2,20,23,33,34). MK-CSA, in murine culture systems, has been obtained from a number of cell-line conditioned media but was difficult to detect from a variety of sources in vivo. (20,34). Recently, a factor has been identified that supports the growth of human MK-CFU (Fig 5,6). It is of major interest since it has been identified in the serum of patients with hypomegakaryocytic thrombocytopenia, but not in the sera of patients equally thrombocytopenic but with adequate or increased numbers of recognizable MK. (35-37). This



Effect of the Addition of Serum Samples from 11 Patients with Thrombocytopenia and Diminished Numbers of Marrow Megakaryocytes on Formation of CFU-M-Derived Colonies.

Each solid circle represents the mean of a single experiment performed in duplicate. Open circles represent the means of all studies performed, and clearly demonstrate augmentation of formation of CFU-M-derived colonies by the serum samples tested ($P<0.0005$).

Figure 5, Reference 35



Effect of the Concentration of Serum from Two Representative Patients Each with Thrombocytopenia Associated with Diminished Megakaryocytes (Open Circle and Solid Square), Thrombocytopenia Associated with Normal or Increased Megakaryocytes (Solid Circle and Triangle), and Thrombocytosis (Open Square and Triangle) on Formation of CFU-M-Derived Colonies.

Each line is derived from a single experiment performed in duplicate. A significant proportional dose-response effect was demonstrated in both patients with thrombocytopenia and diminished megakaryocytes ($R = 0.981$, $P<0.01$ and $r = 0.854$, $P<0.01$). Serum samples from each of the two patients in the other two categories did not alter formation of CFU-M-derived colonies at the doses of serum tested.

Figure 6, Reference 35

MK-CSA stimulates an increase in MK-CFU with a dose-response relationship as well as increasing the number of MK per colony, also in a dose-related fashion. Since, in vivo, the majority of MK-CFU are not in cell-cycle, it may be that this factor acts by inducing cells to enter a proliferative phase. The fact that it cannot be detected in thrombopenic states in which MK are not reduced suggests that its production may be mediated by the size of the MK mass, perhaps as a cell-cell interaction phenomenon. Recently, this MK-CSA has been purified to presumed homogeneity. (38,40). It is aglycoprotein of an apparent molecular weight of 46,000 and has specificity for stimulating MK-CFU with no activity on erythroid or granulocyte-monocyte colony growth.

These observations regarding control of megakaryopoiesis and thrombopoiesis have led to the proposal of the two-stage model of regulation of platelet production. (2) (Fig. 1).

THROMBOKINETICS AND KINETIC CLASSIFICATION OF THROMBOCYTOPENIC STATES

Utilizing 51 Cr labeled platelet survival in conjunction with a quantitation of megakaryocyte numbers, mass and nuclear lobe count, Harker established data on normal platelet production, relationship to recognizable MK characteristics and patterns of alteration in various thrombocytopenic and thrombocytotic states in man. (18,41). Unfortunately the methodology is not applicable to routine clinical assessment of altered paltelet numbers. Recently, however, automated hematology instruments have allowed the application of these kinetic principles to the evaluation of clinical numerical platelet disorders. Table 3 summarizes normal MK-platelet values. An important observation was that there is a linear correlation between mean MK ploidy and mean MK volume and that effective thrombopoiesis is in turn directly related to MK volume. (Figure 2). Recent studies have extended these observations to demonstrate that mean platelet volume (MPV) is linearly

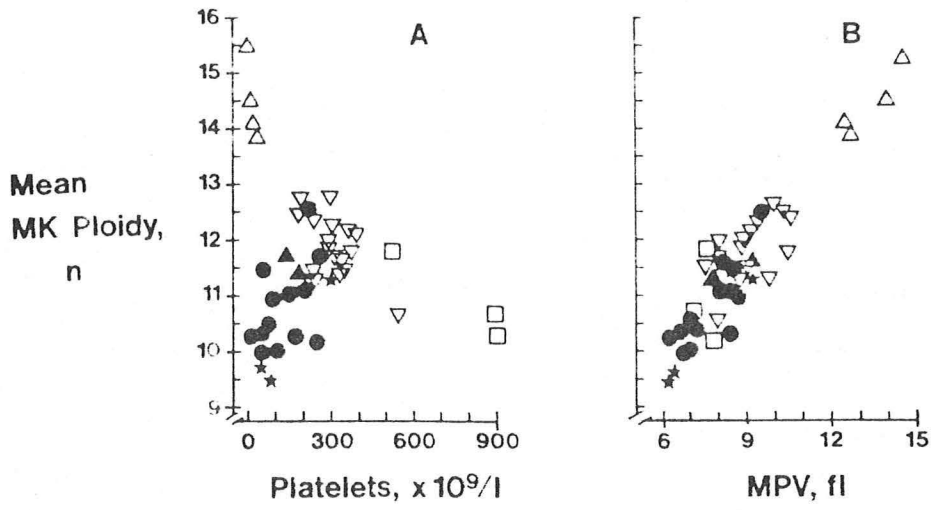
Normal Thrombokinetics in Man

PARAMETER	VALUE (mean ± 2SD or range)
Platelet Count	250,000/μl (150,000 - 399,000)
Mean Platelet Volume (MPV)	Dependent on Platelet Count *
Platelet Survival	9.9 ± 1.2 days
Recovery	64.6 ± 8.2 % **
Platelet Production	35,000 ± 4,300/μl/day
MK Number	6.1 ± 1.4 x 10^6/Kg***
Mean MK Ploidy	12.2 N
Cell Volume/Nuclear Lobe	520 ± 40 μ^3
MK Mass	2.8 ± 0.6 x 10^{10} μ^3/kg
Production per Nuclear Unit	49 ± 10 platelets

*See Nomogram
**Reflects sequestration of platelets in spleen
***By light microscopy - 1/3 more by fluorescent antibody tagging

Table 3, Reference 18, 42

correlated with mean MK ploidy. (42)(Fig. 7). Thus, MPV can be used as a measure of effectiveness and adequacy of thrombopoiesis. (17,25,40,42-45) generation of some automated clinical cell counters provide a mean platelet volume value routinely. This permits a more sensitive and quan-



Relation of megakaryocyte ploidy to: (A) platelet count; (B) mean platelet volume (MPV). Symbols are the same as in Figure 9.

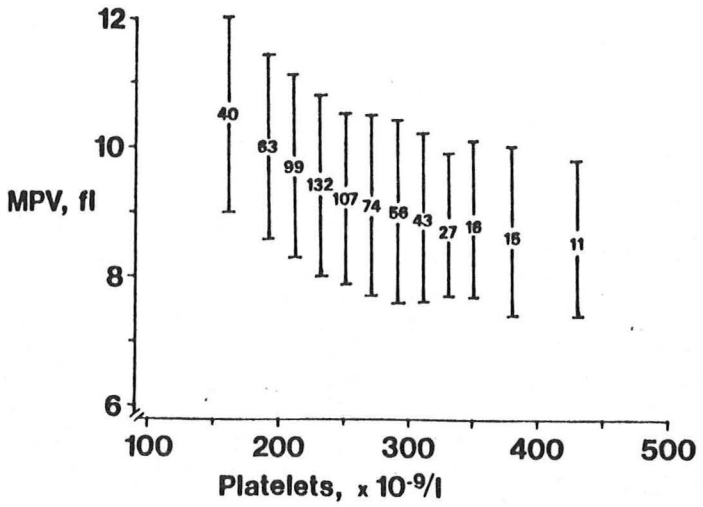
Figure 7, Reference 42

titative measure of thrombopoiesis than the traditional semi-quantitative assessment of MK numbers on bone marrow examination. A simple clinical evaluation of platelet count and MPV (similar to RBC values and MCV), at times in conjunction with a bone marrow examination, allows one to classify mechanisms of thrombocytopenia and thrombocytosis on a kinetic basis. (42-47).

Over the normal range of platelet counts, there is a non-linear variation of mean platelet volume, with increasing platelet numbers being correlated with decreasing MPV. (48)(Fig. 8). As noted above, MPV appears to reflect the mean ploidy of the MK population which in turn is primarily modulated by TSF. In circumstances in which adequacy of MK precursor proliferation and maturation exist, modulations of peripheral platelet numbers would be reflected in alterations of MK numbers and ploidy, and in turn, platelet production rates and MPV. Alternatively, defects in MK precursor numbers or proliferation or abnormal MK nuclear maturation would be expected to result in a pattern of failure of these physiologic responses to an altered platelet mass. (Tables 4 and 5).

DESTRUCTIVE THROMBOCYTOPENIAS

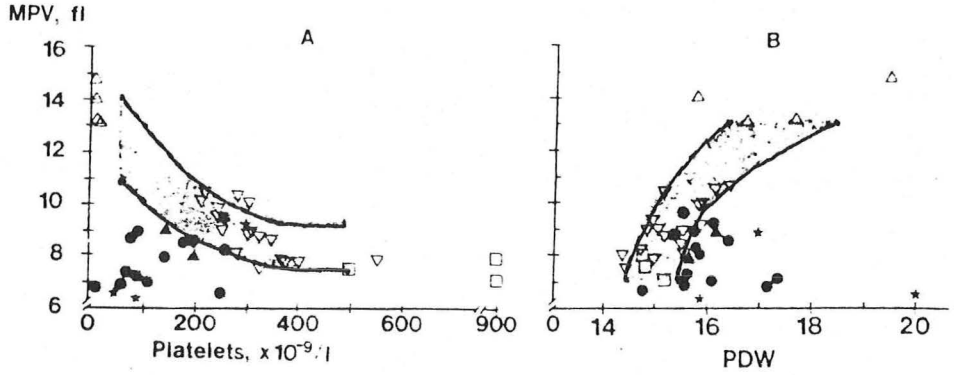
These disorders result from a reduction in the effective life span of circulating platelets secondary to a peripheral mechanism. (49). These pathogenetic processes of reduction of the circulating platelet mass are followed, acutely, by the production of TSF. The resultant physiologic response is to stimulate increased MK ploidy and volume, increased MK mass and therefore increased production of platelets with an increase in mean size. (18,42). Frequently, increased MK numbers also occur, either on the basis of increased commitment to the maturational pool or, later, due



Relation of platelet count and platelet volume in 683 normal subjects. Each group is shown as the mean (number) ± 2 S.D. (bar) of all subjects grouped by platelet counts of: 128–179, 180–199, 200–219, 220–239, 240–259, 260–279, 280–299, 300–319, 320–339, 340–359, 361–403, and 406–462 × 10⁹/l. The number at mean position shows how many subjects were in the group. The statistical difference in MPV among the different groups is shown below (groups are identified by mean platelet count).

		Platelet count, ×10⁹/l											
		160	190	210	230	250	270	290	310	330	350	380	430
Statistical difference ($P <$ ____) from mean value for MPV in patients with platelets of (×10⁹/l)	160	—	N.S.	.05	.01	.01	.01	.01	.01	.01	.01	.01	.01
	190		—	N.S.	N.S.	.05	.02	.02	.01	.01	.01	.01	.01
	210			—	N.S.	N.S.	.05	.02	.02	.01	.01	.01	.01
	230				—	N.S.	N.S.	.05	.02	.01	.01	.01	.01
	250					—	N.S.	N.S.	N.S.	.02	.05	.02	.01
	270						—	N.S.	N.S.	.05	N.S.	.05	.02
	290							—	N.S.	N.S.	N.S.	.05	.02
	310								—	N.S.	N.S.	N.S.	.05
	330									—	N.S.	N.S.	N.S.
	350										—	N.S.	N.S.
	380											—	N.S.

Figure 8, Reference 48



Relation of mean platelet volume to (A) platelet count and (B) platelet distribution (PDW). The hatched area in each panel shows the relation of mean platelet volume to platelet count or PDW in normal subjects [14]. Closed figures represent subjects with presumed impaired megakaryocytopoiesis. Nine had normal platelet counts, yet only three had MPV within the range of MPV in normal subjects with similar platelet counts. (●) Acute myelogenous leukemia (13 patients). (▲) megaloblastic anemia (2). (✱) aplastic anemia (3). Open figures represent subjects with presumed normal or stimulated megakaryocytopoiesis: (△) Immune thrombocytopenic purpura (4); (▽) normal (17); (□) reactive thrombocytosis (3).

Figure 9, Reference 42

THROMBOKINETIC PATTERNS IN THROMBOCYTOPENIC STATES

Parameter	Destructive	Hypoproliferative	Ineffective	Distributional
MPV*	"Normal"	Decreased	Decreased	Decreased
Platelet Survival	DECREASED	Normal	Normal	Normal
Recovery	Normal	Normal	Normal	DECREASED
Production	INCREASED	DECREASED	DECREASED	INCREASED
MK Number	Increased	Decreased	Increased	Increased
Mean MK Ploidy	Increased	Decreased	Decreased	Increased
Vol/Nuclear Lobe	Normal	Normal	Normal	Normal
MK Mass	Increased	DECREASED	INCREASED	Increased
Production per Nuclear Unit	Normal	Normal	DECREASED	Normal

* Relative to Platelet Count

TABLE 4

ETIOLOGIC MECHANISMS OF ACQUIRED THROMBOCYTOPENIA

I Peripheral Destruction - Loss

A. IMMUNOLOGIC

1. Anti-Lymphocyte Globulin

2. Drug Induced

a. Hapten-Immune Complex
b. Heparin Associated

3. Post Transfusion Purpura

4. Acute ITP

5. Chronic ITP

a. Primary
b. Secondary

B. CONSUMPTION

1. Disseminated Intravascular Coagulation (DIC)

2. Thrombotic Thrombocytopenic Purpura (TTP)

3. Hemolytic Uremic Syndrome

4. Platelet Loss
Massive Blood Loss
Exchange Transfusion
Extracorporeal Perfusion

TABLE 5a

II Hypoproliferative

A. APLASTIC ANEMIA

1. Idiopathic
2. Drug (dose dependent and idiosyncratic)
3. Ionizing Radiation
4. Paroxysmal Nocturnal Hemoglobinuria (PNH)

B. SELECTIVE AMEGAKARYOCYTIC THROMBOCYTOPENIA

1. Drugs
2. Alcohol
3. Idiopathic (possibly immune)

C. VIRAL INFECTIONS

D. MYELOPTHISIC STATES

E. MYELODYSPLASTIC SYNDROMES

F. SEPSIS (without DIC)

TABLE 5b

III Ineffective Thrombopoiesis

A. NUTRITIONAL MEGALOBLASTIC ANEMIAS

B. DIGUGLIELMO'S SYNDROME

C. MYELODYSPLASTIC SYNDROMES

IV Distributional Thrombocytopenia (Hypersplenism)

A. CONGESTIVE SPLENOMEGALY

B. MYELOPROLIFERATIVE SYNDROMES

C. LYMPHO-RETICULAR NEOPLASMS

D. RETICULOENDOTHELIOSES

V Pseudothrombocytopenia

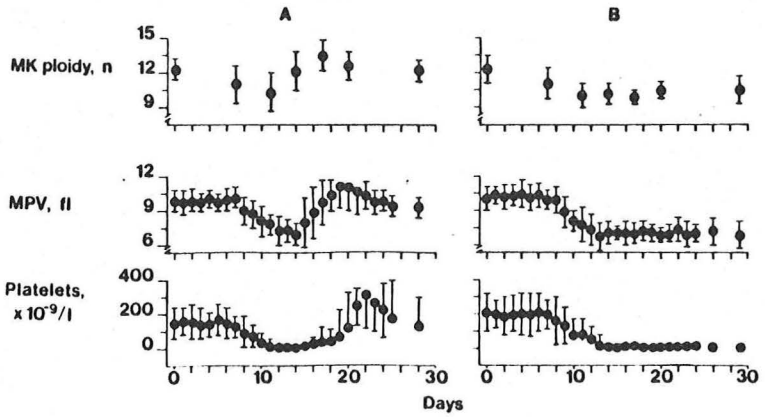
TABLE 5c

to increased precursor proliferation. However, a documentation of increased MK-CFU in these states in humans has not been forthcoming. There is some data to suggest that there may be increased production of MK-CSA resulting in an increase in the proportion of precursors that are in the DNA synthesis stage of the cell cycle. (39). Clinically, these states are characterized by an MPV that is normal for the level of thrombocytopenia, that is, a proportional increase for the decrease in platelet count. (42)(Fig. 9). Bone marrow megakaryocytes will be normal to increased in number. (18). As noted, experimental studies confirm the increase in MK number, MK mass and MK ploidy as well as the resultant increase in platelet production rates. Subsequent clinical assessment requires the appropriate studies necessary to delineate the various mechanisms listed in Table 5. (50-60).

HYPOPROLIFERATIVE THROMBOCYTOPENIA

This group of disorders result from the failure of normal MK precursor commitment or proliferation and thus a reduction in the number of maturing and platelet producing MK. (61). Although there is evidence (particularly in rodents) that the reduced platelet mass results in increased TSF production, the maturing MK do not reflect their presence, either because of defective response of the reduced maturation pool or because premature platelet production occurs at the earlier stages of MK differentiation, or both. (18,42). Clinically, the states are characterized by decreased MPV relative to the circulating platelet count and decrease MK numbers in the bone marrow. (42)(Table 4). Experimentally one finds decreased MK-CFU, decreased MK numbers, MK mass and MK ploidy as well as the resultant decrease in MPV and platelet production. (18)(Fig. 9). Human MK-CSA is increased in these conditions and can be demonstrated to appear rapidly after an insult such as chemotherapy which impairs platelet production. (35,40). Of interest, in this model, MK-CSA may also be under the control of the circulating platelet mass since platelet transfusions transiently suppress its measurable activity. (40).

The relationship between effective platelet production and evidence of response to TSF can also be utilized in the clinical setting to provide early evidence of platelet production recovery after transient chemotherapy suppression. (Fig 10). MK ploidy increases preceed evidence of rising platelet counts by approximately 96 hours and increasing MPV preceeds rising



Summary of serial measurements of megakaryocyte ploidy, MPV, and platelet count. Each value is mean (dot) ± 1 S.D. Chemotherapy given days 2-6. A) The courses followed by recovery. Sequential rise of ploidy, MPV, and platelet count. Megakaryocyte ploidy is subnormal on days 7 and 11, and supranormal on day 17 ($p < 0.05$). B) The three courses not followed by recovery. Megakaryocyte ploidy, platelet volume, and platelet count fall and do not recover.

Figure 10, Reference 62

platelet counts by approximately 48 hours. (62). These processes are usually associated with pancytopenia, although selective hypomegakaryocytic thrombocytopenia is occasionally seen. (63-72)(Table 5).

INEFFECTIVE THROMBOPOIESIS

The term ineffective thrombopoiesis defines circumstances in which there is an impairment of production of platelets in the presence of apparently normal numbers of recognizable MK. The prototype of ineffective myelopoiesis is the megaloblastic states. These conditions result from defective DNA synthesis, particularly of cells in the later stages of the proliferative-maturation pools. As might be predicted, in the case of thrombopoiesis, this is reflected by the finding of normal or increased megakaryocyte numbers, but decreased MK ploidy, mass and MPV. (18)(Fig. 9). The impairment of DNA synthesis prevents normal ploidization of MK and thus the normal maturational stages of MK development. This effect can be seen even with normal platelet numbers by the finding of a low MPV relative to the platelet count. (42).

DISTRIBUTIONAL THROMBOCYTOPENIA

As noted above, approximately one-third of the intravascular platelet pool is sequestered in the spleen but in equilibrium with the circulating pool. (18). When the splenic volume increases, there is often an increase in the poportion of the intravascular platelet pool which is sequestered in the spleen (hypersplenism). (73)(Table 4). Nevertheless, the platelet survival is normal. The fact that the platelet production rate is increased in these patients substantiates the dependence of thrombopoiesis upon the circulating platelet mass. Kinetically, the MK number, mass and ploidy are increased as evidence of an attempt to compensate for this sequestration. Parodoxically, the MPV is low relative to the platelet count. (74). This seemingly inconsistent relationship between effective platelet production and MPV is explained by the fact that the spleen tends to selectively sequester large platelets. Thus the diagnosis of this condition must rely on the recognition of this pattern of thrombopoiesis. The presence of a large spleen in association with adequate BM MK, the low MPV and the lack of evidence of megaloblastic dyspoiesis allows for confirmation of the diagnosis. (Table 5).

PSEUDOTHROMBOCYTOPENIA

The preceding discussion regarding mechanisms of thrombocytopenia assumes that the circulating platelet count is in fact reduced. Several circumstances exist, however, in which the clinically determined platelet count is erroneously low. This event, termed pseudothrombocytopenia or spurious thrombocytopenia, can be defined as a low platelet count resulting from laboratory artifact. (75). This problem is more than a technicalinuisance since a number of patients have undergone extensive diagnostic evaluations for mechanisms of thrombocytopenia and treatment with corticosteroids and splenectomy has been initiated. (76-78). Mechanisms by which these in vitro artifacts are listed in Table 6. The overall incidence of these problems is unclear. They appear to be present more frequently in patients with defined clinical illnesses, especially those with malignant diseases and immunologically mediated disorders. (75,77,80,84). Prospective studies suggest that 0.1-

0.2% of routine blood samples will demonstrate these phenomena. (75,85). The incidence prospectively, in hospitalized patients, exceeds 2%. (75). Of more clinical relevance, Pegels, et al noted that 10 of 500 patients with the diagnosis of ITP actually had this disorder. (78). The frequency relative to true thrombocytopenia is unknown.

MECHANISMS OF PSEUDOTHROMBOCYTOPENIA

Mechanism	References
Platelet Satellitosis	79,80
Platelet Cold Agglutinins	76,80,81
Giant Platelets	75,80
EDTA Dependent Platelet Agglutinins	75,77,78,82-84
Improper Sample Handling	75

Table 6

EDTA Dependent Platelet Agglutinates. More than 90% of instances of pseudothrombocytopenia are a result of platelet agglutinins that are dependent on the presence of EDTA or closely related anticoagulants (EGTA or DPTA) for their activity. In these persons, platelet counts performed on EDTA anticoagulated blood will be significantly lower than the true circulating platelet count, generally ranging from 10-95,000/ul with a mean of 30,000. (73,83,84)(Table 7). Platelet counts performed on blood anticoagulated with citrate, oxalate or heparin will be essentially normal in these subjects. Peripheral blood smears prepared from EDTA blood will demonstrate large platelet clumps, whereas the platelets will be distributed normally on

Serologic Data of All Patients

Clinical Diagnosis	Number of Patients	Positive Indirect Immunofluorescence		Platelet Count × 10^9/Liter			
				EDTA		Capillary	
		EDTA Platelets	Citrate Platelets	Mean	Range	Mean	Range
Pseudothrombocytopenia	10	10/10	3/10*	36	10-70	250	130-365
Idiopathic thrombocytopenia	10	10/10	0/10	35	10-80	204	120-320

*Due to EDTA-independent platelet cold antibodies.

Table 7, Reference 78

direct smears or smears prepared from other types of anticoagulated specimens. (75,77,78,82-84). The agglutination is partially temperature dependent, occurring to a greater degree below 37°C., and is almost immediate after collection of the specimen. (77,78,83,84). The agglutination is independent of platelet aggregation reactions and will occur with aspirin treated platelets. (77). The agglutinating factors have been demonstrated to be antibodies, most commonly of IgG type but also IgM and IgA classes have been reported. (77,78,84,48). In several instances, only a single heavy and light chain

type have been found, suggesting possible monoclonality in some cases. (78). These antibodies are absorbed by platelets only in the presence of EDTA, but do not react directly with EDTA alone. The antibody specificity is not clear, but platelets from persons with Glanzmann's thrombasthenia, which lack the membrane glycoproteins II_B/III_A, do not agglutinate in test systems. (78). The best hypotheses are that either EDTA, by reacting with the platelet membrane, exposes neo-antigens to which these antibodies are directed, or that the antibodies cross react with EDTA acting as a hapten-like substance when bound to the platelet. The diagnosis of this disorder is made by either comparing platelet counts from EDTA anticoagulated blood to samples collected in citrate etc, or by examining a blood smear from EDTA blood for clumping, or smears from other sources for platelet enumeration. It is paradoxically of interest that EDTA is the most commonly chosen anti-coagulant for routine blood counts since, in the absence of these agglutinins, it is the best agent for preventing in vitro platelet aggregation and falsely low platelet counts. Platelet satellitosis appears to be a rare subset of EDTA dependent platelet agglutination. In this circumstance, also EDTA dependent, instead of the platelets forming large clumps, they adhere to granulocytes. This phenomenon is absolutely dependent on the presence of the patient's platelets, granulocytes, and EDTA plasma. (79,80). Platelet cold agglutinins are rare. These are temperature dependent (less than 37°C) antibodies which cause platelet clumping and spuriously low platelet counts in blood collected into any anticoagulant. Fresh blood smears will show normal platelet numbers. Smears made from cooled blood specimens will show platelet clumping. These are antibodies of either IgG or IgM subclasses. (76,80,81). Rarely, patients with an unusually excessive number of giant platelets (megathrombocytes) will have a spuriously low platelet count when determined by electronic particle counters since they are excluded from the upper size threshhold of the instrument. Most commonly, this has been observed in patients with myeloproliferative disorders. (75,80). Of course, blood specimens improperly collected such that clot formation occurs in the tube may appear to have low platelet counts because of platelet aggregation and incorporation into the clot. (75).

In summary, clinically performed platelet counts may occasionally be spuriously low due to a number of laboratory artifacts. All of these mechanisms are physiologically irrelevant since the circulating platelets are unaffected and platelet function is normal. Nevertheless, any apparent instance of thrombocytopenia must have pseudothrombocytopenia excluded prior to diagnostic evaluation and therapy. A number of techniques of identification are available, but the simplest and most reliable is the examination of a freshly prepared, finger-stick peripheral blood smear.

HEPARIN ASSOCIATED THROMBOCYTOPENIA (HAT)

The administration of the anticoagulant heparin may be associated iwth the development of sometimes severe and often persistent thrombocytopenia. (51,86). Several features make this entity atypical for drug-induced thrombo-cytopenic states including the potential for developing severe and often life threatening paradoxical thromboembolic events. Difficulties in the diagnosis and management of this phenomenon have been the subject of several studies during the past few years.

Thrombocytopenia occurring in patients while on heparin therapy appears to represent a heterogeneous group of processes in terms of mechanisms and clinical significance. A slight, immediate and short lived fall in the platelet count with heparin administration has been long known. This may be an in vivo reflection of the ability of certain heparin preparations to produce in vitro platelet aggregation, and is usually not recognized unless a platelet count is performed immediately after a heparin bolus. (51,87). Some patients will develop thrombocytopenia, usually mild, during the first four days of heparin therapy. Often this reverses with continued heparin administration. (86,88). Of potentially greater clinical significance is a more delayed onset and often more severe degree of thrombocytopenia which may be accompanied by arterial and venous thromboembolic events. (51,86,89). Whether this process has any relationship to the earlier onset platelet decrement is unclear. The delayed onset disorder, herein termed HAT, will form the basis for the remainder of the discussion.

The incidence of HAT is uncertain. It may occur with any dose or route of administration. Prospective studies have indicated a frequency ranging from 0-30%. (51,86). Earlier observations tended to indicate a higher frequency that later studies. Criteria in terms of platelet count and time of onset have varied. A more conservative view would be in the range of 5%. There may be some difference in incidence depending on sources of heparin. Four randomized prospective studies comparing bovine vs. porcine heparin indicated a frequency of 10.8% overall with 6% in patients receiving porcine heparin and 15.6% receiving bovine heparin. (86). The severity of HAT also is variable with platelet counts ranging from <10,000/ul to only slightly decreased. The more severe levels have usually been reported in retrospective series. However, the heparin was usually discontinued early after onset of thrombocytopenia in the prospective studies. (89-91). The complications associated with the thrombocytopenia generate the greatest concern. Actually, bleeding manifestations are rather uncommon and are more often associated with excessive heparin dose rather than the presence of HAT. (86,92). The most dangerous associated clinical events are the development of paradoxical thromboembolic problems (TE). The observation that some patients receiving heparin developed arterial thromboemboli was actually made prior to the recognition of the association with thrombocytopenia. (93). It is now clear that arterial and venous thrombi developing in patients on heparin are part of the clinical spectrum of HAT. (51,86,89). Arterial TE are approximately three times more common that recurrent or progressive venous TE. (86). They consist of peripheral extremity arterial and venous thromboses, myocardial infarction, cerebral vascular accidents and pulmonary emboli. (51,86,89,91). The frequency is unknown. Over 50% of retrospectively

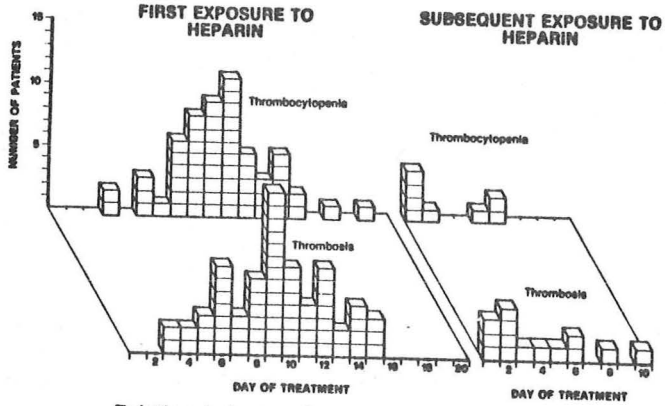
Outcome of Patients with Heparin-Associated Thrombocytopenia and Thrombosis*

	Patients ($n = 85$)
	n(%)
Site of thrombosis	
Arterial	58
Venous	20
Outcome	
Resolution	29
Amputation †	18 (21)
Death	25 (29)

* Data taken from references 1, 22, 33, 35, 37-41, 43-55.
† Some of the patients who underwent amputation subsequently died.

Figure 11, Reference 86

reported cases of HAT have had TE. None have occurred in prospective studies. However, heparin has usually been discontinued early in the development of thrombocytopenia in the latter studies. Almost no cases of TE have been seen unless the platelet count has fallen to below 60,000/ul. (89-91). The TE events may be catastrophic. Twenty-nine percent of patients with HAT and TE have expired and 21% have required limb amputation. (86,89)(Fig. 11). The appearance of HAT occurs at a mean of 9-10 days after starting heparin with a range of 4-22 days. (86,89,91,94)(Fig. 12). Onset of TE is approximately at the same time. However it may be seen almost immediately



Relation between the day that thrombocytopenia or thrombosis was first documented and the day after the start of heparin therapy. Each box represents a different case compiled from references 19, 23, 33-55. Both heparin-associated thrombocytopenia and heparin-associated thrombocytopenia plus thrombosis have a peak incidence at 6 to 12 days after initiation of treatment. Thrombocytopenia or thrombocytopenia plus thrombosis occurs earlier if the patient has had a previous exposure to heparin.

Figure 12, Reference 86

if heparin is restarted in patients who have received the drug within the previous few weeks to month. (86)(Fig. 12). Another atypical feature is that in some persons in whom HAT has been observed, spontaneous recovery of the platelet count on continued heparin therapy has occurred. With the initial discovery that patients with HAT had a factor in their plasma or serum capable of inducing in vitro platelet aggregation in the presence of heparin (platelet aggregation factor, PAF), an immunologic mechanism has been proposed. Initially, some investigators thought that HAT was a mainfestation of DIC. (95). Subsequent investigations have found little or no evidence of DIC in the majority of patients with HAT. (89,94). The ability to identify PAF in patients with HAT varies. Overall, in the literature, about 71% of patients with HAT have an identifiable PAF with the frequency in individual studies ranging from 36 to 100 percent. (86,88,90,91). Much of the variation may be technical as well as the fact that during the acute phase, the PAF may be totally bound to patient platelets and not appreciated when normal platelets are used in the test system. Despite the uncertainty of the sensitivity of PAF assays, the specificity seems to be excellent since less than 5% of patients receiving heparin without HAT or with other causes of thrombocytopenia have a positive test for heparin dependent PAF. (88,90,94). A number of other observations support the concept that HAT is immunologically mediated. The time of onset is consistent with an immunologic

mechanism. Rechallenge of patients with heparin who have had HAT results in an almost immediate fall in the platelet count. (86,89,91). Up to 100% of patients with HAT have increased platelet associated immunoglobulin (PAIg)(90,96). This is not a specific finding since patients with a number of other imunologic, and perhaps non-immunologic causes of thrombocytopenia also have elevated levels of PAIg. (90,97). Finally, PAF has been isolated from the Ig fractions of serum from patients with HAT. It has usually been an IgG but occasionally an IgM molecule. Binding studies suggest that heparin may be acting as a hapten in association with the platelets. (92,94,96,98-101). Unlike other drug induced platelet antibody processes, however, the identifiable PAF disappears from the serum much earlier, usually by 4-5 months. (88,90-92,100).

The approach to the diagnosis and management of HAT is debated. Since the frequency is low and a transient thrombocytopenia of different mechanisms may occur with heparin administration, the cost-effectiveness of serial platelet counts is uncertain, but such an approach has been advocated. (86,89,91). Profound thrombocytopenia with TE is uncommon, but may be catastrophic. Platelet count monitoring with discontinuation of heparin when the count is approaching 60,000/ul might prevent most of these cases. Once thrombocytopenia is recognized, if a PAF assay is available and is positive, it is a highly reliable indicator of HAT. However, a negative study does not exclude this phenomenon. Other causes of thrombocytopenia must be considered, including other concomittantly administered drugs and DIC. In the presence of moderately severe thrombocytopenia, alternate means of anticoagulation should be strongly considered. In the presence of thrombocytopenia and new or progressive TE, heparin therapy should be discontinued. (86,89,91). Recovery of the platelet count occurs with a mean time of 5-6 days with a range of 1-20 days. (86,89,94).

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