STUDIES ON HUMAN PLATELETS*

VI. STUDY OF PLATELET ABNORMALITIES BY DENSITY GRADIENT SEPARATION OF PLATELET POPULATIONS

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Normal human platelets generally range from 2 to 4 μ in diameter. However, in a number of diseases with associated bleeding tendencies, platelets often appear large, bizarre and amorphous, showing lack of granularity and poor distinction between chromomere and hyalomere.[1-3] Electron-microscopic examination of these atypical platelets shows distinct morphologic changes.[4-6] In addition, various biochemical defects such as reduced glucose consumption, decreased ATP content and a decrease or absence of a number of enzymes have been described in these atypical platelets.[7-9]

Although the platelet count may be within normal limits in a given situation, platelet appearance and function may be grossly abnormal. Examination of the role of qualitative platelet abnormalities in the pathogenesis of some cases of haemostatic failure can and has provided a means of detecting and interpreting some poorly understood but important aspects of haemostasis and platelet function. The evaluation of qualitative platelet defects can, however, prove to be very tedious. The purpose of this communication is to describe a rapid method of monitoring and evaluating alterations in platelet morphology which can be associated with bleeding tendencies.

Human platelets have been separated into 4 populations on a sucrose density gradient.[10-13] These populations have been characterized under the electron-microscope[12,13] and in terms of a number of biochemical parameters such as protein synthetic activity, ATP production, enzyme activity and aggregation properties.[32-34] Based on the known morphologic and biochemical properties of the separated populations, it has become apparent that platelet density gradient patterns can provide a qualitative and quantitative picture of the platelet populations present in various clinical situations.

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MATERIALS AND METHODS

Preparation of Platelets

Whole blood (2·5 - 5 ml.) containing anticoagulant (1 ml. formula A ACD for every 5 ml. whole blood) was diluted with 22 ml. of isotonic saline-ACD solution (20 ml. isotonic saline plus 2 ml. ACD formula A solution). Platelet-rich plasma (diluted) was obtained by centrifugation at 80 g for 35 minutes. The platelet-rich plasma was removed and recentrifuged at 80 g for 15 minutes. Platelets were pelleted by centrifugation at 1,100 g for 15 minutes. No leucocyte or RBC contamination could be detected under the light microscope at this stage.

The platelet pellet was resuspended in 1·0 ml. of isotonic saline-ACD solution (1 ml. ACD formula A solution plus 5 ml. isotonic saline) and applied onto the sucrose gradients in appropriate volumes. The amount of blood required to obtain the platelet samples is dependent on the platelet count. With a normal platelet count, 2·5 - 5 ml. of blood yields an adequate platelet pellet and distribution pattern. With markedly increased or decreased platelet counts, proportionately smaller or larger volumes of blood are required. All procedures were carried out at 5°C.

Gradient Preparation and Quantitation of Separated Platelet Population

Gradients were prepared from solutions containing 30, 50, 55, 58·8 and 65% sucrose (w/v), 2 mM EDTA, 0·15

M NaCl, 10 mM Tris-HCl, pH 7·4 (at 25°C) and were precooled to 5°C before making the gradients. The sucrose solutions were carefully layered on one another in 1·1 ml. volumes in a centrifuge tube (½ in. × 2 in.). The 30% sucrose solution was layered on top of the gradient as a 0·4 ml. volume, giving a discontinuous gradient with 4 density interfaces. Centrifugation was carried out in the cold (5°C) at 21,000 r.p.m. for 45 minutes in the SW50 swinging-bucket rotor of the Spinco Model L centrifuge. The separated platelets (bands A, B, C and D; band D representing the platelet population of highest density) were monitored by displacing the gradient through an ISCO Model UA-2 ultraviolet analyser and recording the output signal on a Sargent SR recorder. A typical distribution pattern of a normal platelet population is illustrated in Fig. 1(a).

Electron-microscopy

Platelets from each of the four populations (A, B, C, D) were prepared for electron-microscopy by fixing in 3% glutaraldehyde for 45 minutes at 5°C, post-fixing with 1% osmium tetroxide, embedding in Epon and staining with lead citrate and uranyl acetate.

RESULTS

Characteristics of Platelet Populations

The effects of inhibition and induction of thrombopoiesis on the platelet distribution pattern are illustrated in Fig. 1(b) and 1(c). Inhibition or suppression of platelet production was obtained in renal transplant patients treated with high doses of the immunosuppressive drug, azathioprine. The decrease in band C and band D platelets (Fig. 1(b)) was associated with a decrease in the average platelet count from 3·5 × 10^5/cu.mm. to 9 × 10^4/cu.mm.

Formation of new platelets was induced in a patient with chronic thrombocytopenic purpura by transfusion with small amounts of normal plasma.[15] When compared with the distribution pattern obtained before transfusion, the pattern obtained following induction showed a marked increase in band C and band D platelets. The increase in band C and band D platelets was associated with a simultaneous increase in platelet count from 2 × 10^4/cu.mm. to 5·2 × 10^5/cu.mm.

The separated platelet populations were characterized under the electron-microscope. A composite electronmicrograph of platelets from each of the 4 bands is shown in Fig. 2. The most pronounced morphologic differences are found in comparing platelets from band A (top of the gradient) with those in band D (bottom of the gradient).

Band A is composed almost entirely of platelet membranes, fragments, and extensively damaged platelets containing very few or no granular structures. In contrast, band D consists of intact platelets of uniform size and shape containing numerous granular structures. Bands B and C appear to represent various intermediate stages between the morphologically distinct populations in bands A and D. We have concluded that bands C and D represent biochemically competent and morphologically intact platelets, whereas bands A and B represent damaged platelets. This conclusion is based on the results of the studies on induction and inhibition of platelet formation.

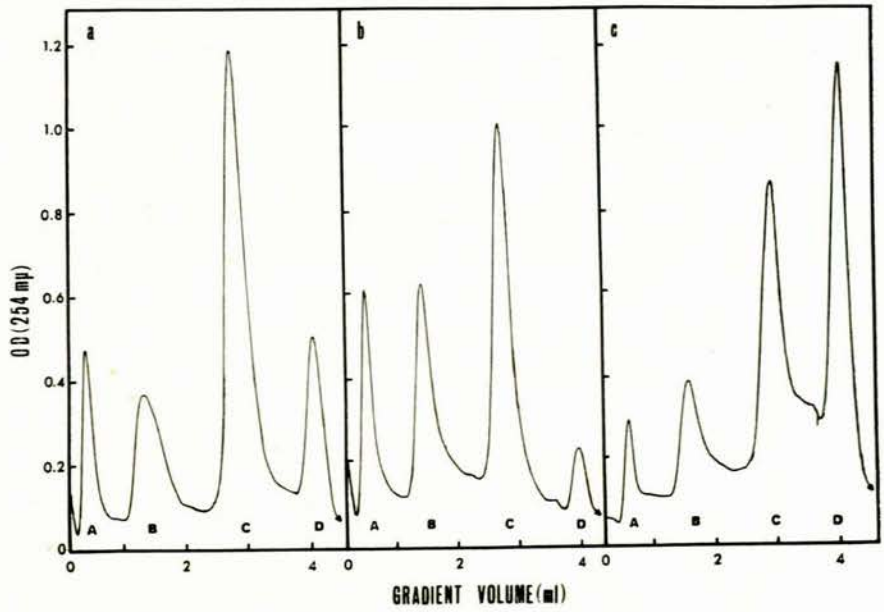

Fig. 1. Separation of human platelets into four populations (A, B, C, D) on a discontinuous sucrose gradient. Arrows indicate bottom of gradients. *a*: Normal individual (platelet count: 2·5 × 10^5). *b*: Individual undergoing immunosuppressive therapy (after renal transplant) in which platelet formation was depressed with azathioprine (Imuran) (platelet count 9·10^4). *c*: Individual with chronic thrombocytopenic purpura in which platelet formation was induced with normal plasma (platelet count: 5·5 × 10^5).

electron-microscope examinations, and biochemical studies on protein synthetic activity, enzyme activity and ability to aggregate.[10-14]

Clinical Studies

The remarkable constancy of platelet distribution patterns in normal individuals (Fig. 1(a)) led us to investigate the application of this technique in studying clinical situations in which platelet abnormalities have been described or suspected. Preliminary results have indicated that a number of clinical disorders with bleeding tendencies were associated with alterations in the distribution of platelet populations. To illustrate the use and application of this technique we will consider only patients with uraemia and polycythaemia vera in which bleeding tendencies have been well documented.[16-19]

Uraemic patients (before haemodialysis) have a markedly altered platelet distribution pattern as is shown in Fig. 3(b). Compared with the normal pattern (Fig. 3(a)), it is evident that bands C and D are virtually non-existent and that there is a significant elevation in bands A and B representing membranes and damaged platelets. It is important to note that haemodialysis of the patients restored the distribution patterns to normal with a simultaneous decrease or disappearance of bleeding tendencies.

An alteration in platelet distribution similar to that found in uraemia was observed in patients with polycythaemia vera (Fig. 3(c)). The most significant feature was again the marked increase in membranes and damaged platelets (bands A and B) and a decrease in intact platelets (bands C and D).

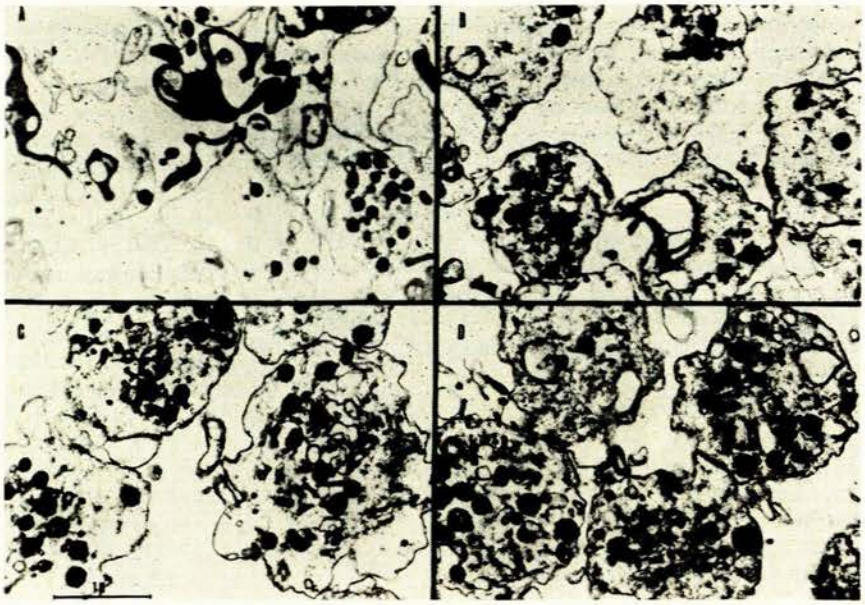

Fig. 2. Electron-micrographs of platelet populations (A, B, C, D) separated on a discontinuous sucrose gradient.

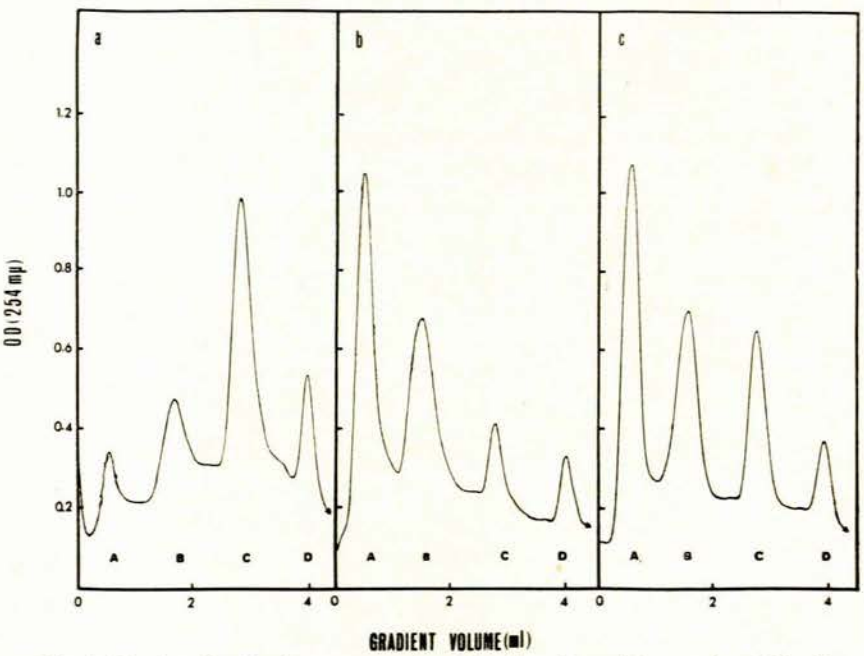

Fig. 3. Platelet distribution patterns found in disorders with associated bleeding tendencies. *a*: Normal. *b*: Uraemia. *c*: Polycythaemia vera.

DISCUSSION

Studies on the distribution of platelet populations in uraemia and polycythaemia vera by means of density gradient analysis have indicated that qualitative defects do exist in these disorders. The reproducibility of platelet distribution patterns in normal individuals suggests that alterations in the steady state level of platelet distribution will produce alterations in the normal distribution pattern.

In vitro studies conducted in this laboratory have shown that band C and band D platelets are most active in aggregation.[11-13] This observation is consistent with Wright's report that newly formed platelets were 'stickier' than those normally present.[21] During aggregation, platelets from band D undergo marked density changes resulting in rapid shifts to bands B and C, and eventually to band A. These data, in conjunction with the finding of damaged platelets in uraemia and polycythaemia vera, indicate that biochemically and morphologically intact platelets are required to initiate platelet aggregation and

subsequent haemostasis.

Platelet distribution patterns of postsurgical patients with transient thrombocythaemia were very similar to the distribution pattern obtained after induction of thrombopoiesis (Fig. 1(c)). Wright reported that new platelet formation caused the increased count in such cases.[20] These findings support the conclusion drawn from our *in vitro* studies which showed that platelet bands C and D are most active in platelet aggregation.[11-13]

In the cases studied, bleeding tendencies were associated with altered distribution patterns in which platelet bands C and D were diminished or virtually absent and platelet bands A and B were markedly elevated. In such cases, platelet bands A and B represented almost the entire platelet population (Fig. 3(b) and 3(c)). Analysis of a large number of distribution patterns from patients in which bleeding tendencies existed indicated that the platelet populations represented by bands C and D have an important role in the haemostatic process and are required in sufficient amounts or ratios to prevent bleeding disorders.

Although platelet counts were within the $1{\cdot}9 \times 10^5$ to $4{\cdot}5 \times 10^5$/cu.mm. range in most cases studied, density gradient analysis showed very definite qualitative alterations. These qualitative alterations reflect changes in platelet density due to changes in size, shape, protein content, granulation and metabolic activity. In both uraemia and polycythaemia vera the platelet populations consisted almost entirely of membranes and damaged platelets (bands A and B). These changes in the distribution of platelet populations may represent platelets with little or no ability to maintain normal haemostasis and may therefore be the major cause of the observed bleeding tendencies.

Although the platelet distribution patterns in various disease states are reproducible, it should be emphasized that the pathophysiologic and diagnostic significance of such alterations remains to be ascertained. Analysis of platelet distribution patterns in conjunction with platelet function studies and changes in platelet counts may provide additional information on the clinical significance of qualitative and quantitative platelet defects.

SUMMARY

Human platelets have been separated into four biochemically and morphologically characteristic populations (bands A, B, C, D) by density gradient centrifugation. Bands A and B contain platelet membranes and damaged platelets; bands C and D contain intact platelets with distinct internal structure. Quantitation of the platelets in each population gives a distribution curve which is virtually identical for normal individuals. The distribution curve is significantly altered in several conditions where platelet abnormalities have been described or suspected. Analysis of platelet distribution patterns provides a qualitative and quantitative method for studying normal platelets as well as clinical platelet abnormalities.

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