Histology of Platelet-Plasma Clots from Normal Subjects and Patients with Abnormal Coagulation

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With the remarkable advances in our knowledge of the physiology and biochemistry of blood coagulation in recent years, little attention has been directed at the morphology of blood clots. Notable exceptions have been the work of Benthaus and of Conley and his colleagues, who have studied the structure of normal platelet-fibrin clots and the effect on structure by alteration of chemical constituents of blood; Benthaus includes a particularly comprehensive review of earlier studies.

These studies were undertaken to examine the morphology of platelet-plasma clots from normal subjects and to compare these to clots from patients with various abnormalities of coagulation.

Methods and Material

Whole blood clots are so filled with erythrocytes that the finer morphology of the clot is obscured. It was therefore necessary to employ platelet-plasma clots, admittedly neglecting the possible role of the erythrocyte in coagulation.

One hundred ml. of whole blood were drawn into a plastic bag containing ACD anticoagulant. The blood was then transferred to 100 ml. Lusteroid centrifuge tubes and placed in a refrigerated centrifuge at 5°C. The first centrifugation was critical, involving the separation of platelets from the other formed elements of the blood by differential centrifugation. The blood was centrifuged for 15 minutes at 150 to 200 g.

The plasma obtained from this slow centrifugation contained most of the platelets. After this plasma was removed, the platelet yield could be increased by centrifuging the remaining whole blood again at the same speed for 15 more minutes and pooling the plasma obtained from both centrifugations.

The platelet-rich plasma was then spun for 30 minutes at 2000 g. The platelet-poor plasma was decanted and saved. The sediment of platelets was then resuspended in approximately 80 ml. of normal saline, centrifuged at 2000 g for 20 minutes, and the saline discarded. The procedure was repeated three times, to wash the plasma from the surface of the platelets.

After the last washing, the platelets were again resuspended in saline and a platelet count was done with a phase microscope. The desired stock concentration of washed platelets was 2,000,000/cu. mm., and the diluent was adjusted to provide a mixture of that concentration.

To prepare clots the following mixture was employed: 1.8 ml. of platelet-poor plasma + 0.2 ml. of platelets of a predetermined concentration (20 thousand to 2 million per cu. mm.) + 0.2 ml. of highly purified thrombin (100 units/ml.). Immediately after the

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thrombin was blown in, the tubes containing the mixture were placed in a 37 C. water-bath. To obtain clots at various intervals after the onset of coagulation, the process was stopped by transferring the contents of the tubes to a wide-mouthed jar and quickly adding 10 per cent neutral formalin. Clots for sectioning were obtained in this manner at 30 seconds, 1 minute, 2 minutes, 5 minutes, 10 minutes, 30 minutes and 18 hours.

After fixation in formalin, the clots were placed in 70 per cent alcohol for two to three days, the larger clots for the longer period. They were then transferred every four hours to graduated concentrations of alcohol (70 per cent to 80 per cent to 95 per cent to absolute). The dehydrated clot was then cleared in two changes of xylene for two hours per change, and impregnated in three changes of paraffin for one hour each. This was then imbedded and cut into sections of approximately six μ thickness. Certain clots were stained with eosin or acridine orange, and studied under conventional and ultraviolet light with straight transillumination as well as a phase condenser, but the most useful stain proved to be Mallory's phosphotungstic acid hematoxylin (PTAH), examined either with directly transmitted light or phase illumination.

Following the determination of the appearance of normal clots at various periods after onset of clotting, clots were prepared from patients with the following diseases: Polycythemia vera, accelerator (AC) globulin (Factor V) deficiency, macroglobulinemia of Waldenström, plasma thromboplastin component deficiency (Christmas disease or hemophilia B), and fibrinolytic syndrome in a patient with carcinoma of the prostate. Selection of these disease states was made to compare conditions which alter coagulation due to presumed platelet abnormalities and those which alter coagulation due to presumed plasma abnormalities. Three patients anticoagulated with bishydroxycoumarin were also studied for comparison to both the normal and abnormal clots.

To obtain clots with normal histologic appearance consistently, the following factors are required: (1) Both platelets and plasma (in volumes as indicated above) from a normal donor; (2) platelet concentration of 200,000/cu. mm.; (3) atraumatic handling of the platelets; (4) addition of highly purified preformed thrombin. In normal blood, a normal appearing clot could be obtained simply by recalcification with calcium chloride or by adding preformed thrombin. In blood from patients receiving dicumarol, a normal appearing clot could be obtained by adding calcium chloride and thromboplastin, but the time of normal appearance was delayed longer than if preformed thrombin was added. Except in the patient with PTC deficiency (see below), the preparations from patients with abnormal coagulation were all formed by addition of preformed thrombin, thus removing any variable which defective prothrombin conversion may have otherwise introduced.

**Results**

Normals. The appearance of clots formed from normal plasma and platelets (200,000/cu. mm.) is distinctive, exhibiting a remarkable geometric pattern (fig. 1). The first appearance of this pattern was usually not apparent until the 5-minute preparation and earlier sections proved of little value. By 10 minutes the organization was complete, with platelet agglomerations acting as focal points for the construction of a coarse fibrin lattice. These coarse strands of fibrin connecting one group of platelets with all neighboring groups formed the major structure, but in the intervening spaces there was a homogeneous, fine network. With passage of time, clots contracted, making the structure more coarse and bulky.

Clots made with low platelet concentration (20,000/cu. mm.) were generally normal in appearance, but the lattice construction was loose (fig. 2). Clots made with high platelet concentrations (2,000,000/cu. mm.) were difficult to evaluate. The platelet agglomerates were so numerous that the precise form of the lattice construction could not be determined. It was apparent,
Fig. 1.—Three photomicrographs of platelet-plasma clots from normal subjects. All are stained with PTAH, magnified 160x (reduced), and were formed with a platelet concentration of 200,000 cu. mm. by adding preformed thrombin. A is from a clot 10 minutes old, B 30 minutes and C 18 hours.

however, that abnormally high concentrations of platelets were associated with defective formation of the fine-mesh network (fig. 3).

Polycythemia vera. Coagulation in this disease is abnormal and serious bleeding sometimes occurs. The nature of the defect in coagulation is not clear, but there is evidence of abnormal function of the platelets, which are often present in concentrations much greater than normal. In studying the phenomenon of “red cell fallout” from the clots of patients with polycythemia...
Fig. 2.—These photomicrographs demonstrate the effect of decreasing the normal platelet concentration, representing “Thrombocytopenia.” Since both platelets and plasma are normal here, they are not representative of the clinical entity of idiopathic thrombocytopenic purpura. All are stained with PTAH and magnified 160x (reduced). In A to C the platelet concentration is 20,000/cu. mm. and sections were made 10 minutes, 30 minutes and 18 hours after the onset of clotting.

vera, we noted that the major morphologic abnormality was the presence of large gaps in the fine network of fibrin and suggested that red cells escaped through these holes instead of being normally enmeshed.

Five patients with this disease have now been studied with the technics described. In each, the same abnormality of the fine network of fibrin was apparent (fig. 4A-B). In addition, the coarse strands of fibrin were not uniform, varying greatly both in diameter and length; this was particularly
Fig. 3.—These three photomicrographs represent "thrombocytosis," the clots being made with 2,000,000 platelets/cu. mm. All are stained with PTAH and magnified 160x (reduced); A, B, and C are at 10 minutes, 30 minutes and 18 hours, for comparison with figs. 1 and 2. Reasons for the clot disorganization by excessive platelets is uncertain. Possibly construction of the fine-mesh network is disrupted when more than the usual number of platelets compete for fibrin in forming the coarse network. This type of clot deformity due to thrombocytosis may contribute to the bleeding tendency of patients with polycythemia vera, but such patients also form abnormal clots with normal concentrations of platelets (fig. 4).

evident in the later stages of clotting. Since platelet agglomerates formed the nidus for each convergence of coarse fibrin strands, this observation was consonant with electron microscopic studies which indicated abnormal pseudopod formation by the platelets of patients with polycythemia vera.12

The nidus on which the finer strands of fibrin converge was more difficult
Fig. 4.—A and B are photomicrographs of a typical mature clot (18 hours) from a patient with polycythemia vera. PTAH stain; A at 160x and B at 319x (reduced). C is a photomicrograph of a clot formed by combining the platelets of a normal patient with plasma from a patient with polycythemia vera; PTAH stain, 319x, 30 minute clot. Concentration of platelets in all three sections is 200,000 per cu. mm. and the clots were prepared by adding thrombin. Note the size of the erythrocytes (well seen in B) relative to the holes in the fibrin network.

to define. If it was composed of platelets, the finding of large holes in this fine network fit well with the hypothesis of abnormal platelet function, but with the present technic we could not be certain whether there were platelets at all these junction points in the fine network or not. To examine this question further, the platelets of two patients with polycythemia vera were carefully washed and then suspended in the plasma of two normal patients. The washed
platelets of the normal patients were in turn suspended in the plasma of the patients with polycythemia.

Clots formed by combining polycythemia vera platelets with normal plasma were abnormal as expected. There were the same large gaping holes in the fine fibrin network and coarse irregularity of the heavier basic fibrin network as when both platelets and plasma were from the polycythemia vera patient. However, when clots formed by combining normal platelets with plasma from the patients with polycythemia vera were examined, we were surprised to find the same abnormality present (fig. 4C). This suggests that the clotting abnormality in polycythemia vera is not due solely to disordered platelet function but may also be the consequence of some other factor. Further study of this question is indicated.

Macroglobulinemia of Waldenström. If a plasma factor contributes to the abnormal coagulation in polycythemia vera, one might find similar changes in clots from patients with known plasma abnormalities. For this reason, clots were formed (by adding preformed thrombin) from a patient with macroglobulinemia of Waldenström who exhibited the typical mucosal bleeding. The
Fig. 6.—Four photomicrographs of clots from a patient with Christmas disease. All stained with PTAH and composed with 200,000 platelets/cu. mm. The clot in A was formed with calcium chloride but no thrombin or thromboplastin; at 18 hours there is still no organization into normal architecture (160x). B to D are clots from the same patient after the addition of preformed thrombin. B is for comparison with A, both magnified 160x at 18 hours. C and D are a 30-minute clot formed with thrombin from the same patient, magnified 64x and 160x, respectively, to show details of the completely normal architecture. (Reduced.)
same gaping holes in the fine fibrin network were observed as in polycythemia vera; however the network of coarse fibrin strands seemed more orderly (fig. 5). Possibly the coarse network abnormality is determined more by platelet dysfunction, and the fine network malformation by plasma constituents, but this is at present conjectural.

Christmas disease (hemophilia B). To study another form of plasma abnormality, a patient with a deficiency of plasma thromboplastin component (PTC) was chosen. Structure of the clots formed by addition of calcium chloride (but no thrombin or thromboplastin) was grossly abnormal, with neither the fine nor coarse networks of fibrin appearing (fig. 6A). Addition of preformed thrombin to the platelet-plasma mixture of this patient, however, produced the formation of perfectly normal clot structure (fig. 6B-D).

Observations on the clots of this patient were valuable as a demonstration of the expected effects of adding preformed thrombin to the plasma of a patient with this disease. It was equally valuable, however, as a demonstration of the validity of the technic.

AC-globulin deficiency. Clots formed from platelets and plasma of a patient with AC-globulin deficiency by adding preformed thrombin were essentially normal. The network of coarse strands of fibrin were perfectly intact, but the intervening fine meshwork was not completely homogeneous (fig. 7). We are unable to explain why these clots were not completely normal when prepared by adding preformed thrombin. Recalcified platelet-plasma mixtures (without addition of thrombin) failed to form normal clots, just as in the patient with Christmas disease.

Fibrinolytic syndrome. Reasons are apparent for interest in the morphology of clots formed by the platelets and plasma of this patient with carcinoma of the prostate and clinical evidence of massive bleeding. A high titer of a potent circulating fibrinolysin was demonstrated repeatedly.

These clots were abnormal in several ways. In specimens examined only
Fig. 8.—Three photomicrographs of clots formed with platelets (300,000 per cu. mm.) and plasma from a patient with a circulating fibrinolysin and carcinoma of the prostate. A is 1 minute after the onset of clotting; 319x under phase light; B is 5 minutes, 160x, and C at 18 hours, 160x. At 1 minute some of the fibrin strands appear as if cut; at 5 minutes less of this is apparent, and the coarser clot architecture seems normal, though the fine mesh of fibrin is almost absent. At 18 hours virtually all the fibrin network is gone, both fine and coarse, and only a platelet cast remains. (Reduced.)

A few minutes after clotting began, fine strands of fibrin were seen to be broken or attached only at one end, as if they had been cut (fig. 8A). From this point on, the superficial appearance of the clots became somewhat more normal with passage of time (fig. 8B), until the 30-minute and 18-hour specimens. In these
Fig. 9.—Photomicrograph of a clot from a patient anticoagulated with bis-
hydroxycoumarin. The architecture is normal at 30 minutes. Preformed thrombin
was added to prepare these clots; concentration of platelets 200,000/cu. mm., PTAH
stain, 128x (reduced). Similar normal clots were obtained in this patient by adding
calcium chloride and thromboplastin, but the time of appearance was later than
when preformed thrombin was added.

two late specimens, all the fine network of fibrin was lysed, and most of the
course network as well. The remaining “pseudostructure” probably represents
a cast formed by platelets about former fibrin strands which have been dis-
solved (fig. 8C).

Patients anticoagulated with bishydroxycoumarin. Morphology of the clots
of these three patients, formed by adding thrombin, was normal (fig. 9). If
calcium chloride and thromboplastin were added instead of preformed
thrombin, the clot histology was still normal, but its mature appearance was
slightly delayed.

DISCUSSION

These observations offer further evidence of the usefulness of studies of
platelet-plasma clot morphology. The structure of the clots in polycythemia
vera and macroglobulinemia suggest possible mechanisms responsible for the
abnormal coagulation observed in such patients clinically. The evidence that
the abnormal platelet function in polycythemia vera may be secondary is
intriguing, but the observations are too limited to warrant further discussion
at present. The appearance of the fibrin dissolution in one patient with a
circulating fibrinolysin seemed to be biphasic, with an early and then later
peak of activity. All these observations are interpreted from comparison with
the morphology of normal platelet-plasma clots.

Certain pitfalls exist in interpreting the results obtained with the present
method. For example, although the platelet-plasma clot may represent the
structure of the normal whole blood clot, this is presumptive. The possible
active effect of erythrocytes or other elements removed in forming the clots
of this study must be considered. Furthermore, such observations with either
platelet-plasma or whole blood clots are in vitro, and the behavior of the same
blood within a vessel may be quite different. Our attempt to reproduce the
same technic in human blood vessels obtained during surgery has so far
been unsuccessful. Even were such an attempt successful, however, the dif-
ference in behavior of static blood from flowing blood would still remain an
uncontrolled factor.

All efforts were made to handle the platelets gently during the preparation
of the clots. Nevertheless, considerable trauma is unquestionably introduced
during the washing procedure. What effect this may have on either normal or
abnormal platelets remains unknown. Removal of platelets from their “atmos-
phere plasmatique” and its possible alteration of the platelets’ “sponge”
function, are further potential defects in this method. The consistency of a
normal appearance of the clots when it was expected was of some reassurance
in this regard.

Finally, study of the sectioned clot presented certain factors of arbitrary
selection in the production of photomicrographs. Clot sections were sometimes
not homogeneous (especially in the patients with abnormal coagulation),
but the reproduced sections are ones which were considered representative.

Within the limits imposed by the method, we believe there is much to be
learned about both normal and abnormal coagulation from a study of the
morphology of platelet-plasma clots.

SUMMARY

Microscopic study of platelet-plasma clots has demonstrated certain struc-
tural defects in clots from patients with diseases manifest by abnormal
coaulation. Abnormally formed clots were found from patients with poly-
cythaemia vera, macroglobulinemia, Christmas disease, AC-globulin deficien-
cy and fibrinolytic syndrome. Some of the problems imposed by the method are
discussed.

SUMMARIO IN INTERLINGUA

Le studio microscopic de coagulos de plchattas e plasma ha demonstrate
le presentia de certe defectos structural in coagulos ab patientes con morbos
manifeste per coagulation anormal. Anormalmente formate coagulos esseva
trovate ab patientes con polycythemia ver, macroglobulinemia, morbo de
Christmas, deficientia de globulina AC, e le syndrome fibrinolytic. Es discutite
certe problemas imponite per le metodo.

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