Immunocytochemical Studies on Platelets. The Demonstration of a Common Antigen in Human Platelets and Megakaryocytes

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AT THE PRESENT TIME most investigators will accept the morphologic evidence suggesting that platelets originate from the cytoplasm of megakaryocytes.[1,2] The demonstration of a common chemical make-up of platelets and megakaryocytes can be approached by recently developed immunochemical technics. In the present study an anti-human platelet serum was labeled with fluorescein and the conjugated antibody was used as an immunocytochemical stain on human platelets and megakaryocytes according to the fluorescent antibody technic of Coons and Kaplan.[3] The results gave direct evidence for the presence of at least one common antigen in platelets and megakaryocytes. Using a similar approach, Humphrey[4] demonstrated a common antigenic structure between guinea pig platelets and megakaryocytes. The similar antigenic make-up of these cells offers additional support to the original hypothesis of Wright that platelets take their origin from megakaryocytes.

MATERIALS AND METHODS

Preparation and Characterization of Rabbit Anti-Human Platelet Serum

Platelets were isolated by differential centrifugation from 500 ml. units of normal human blood obtained in plastic bags with added EDTA as an anticoagulant. The donors used were of blood groups A, B, AB and O. The platelets obtained from each unit of blood were washed at least three times in 0.85 per cent sodium chloride and finally suspended in saline. Groups of two rabbits were immunized independently with platelets of each of the blood types. Each animal received 10 ml. platelet suspension of the corresponding blood group emulsified in 10 ml. of Freund's adjuvant (without TB bacilli). The emulsion was injected at one time into multiple intramuscular sites. High titer rabbit anti-human platelet plasma was obtained as determined by the platelet agglutinating activity of such plasma. Oxalated plasma rather than serum was used to avoid any contamination with thrombin which can also cause platelet agglutination. No differences in the agglutinating properties were noted in the antiplatelet plasma from the rabbits independently injected with the four types of platelets, i.e., each type antiserum was able to agglutinate all types of platelets and viceversa, therefore the plasma from all rabbits with a high antiplatelet titer was pooled before further fractionation and characterization. The pooled plasma was treated with barium sulphate (100 mg./ml.) and heated to 56 C. for 30 minutes, thus removing or inactivating most of the coagulation factors. A globulin fraction of this

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material was isolated by fractionation with 40 per cent saturated ammonium sulfate. Using continuous curtain electrophoresis[5] it was possible to demonstrate platelet agglutinating activity in the faster moving gamma globulin fraction of this material.

Fluorescent Antibody Technics

Five hundred mg. protein aliquots of the globulin fraction of the antiplatelet serum were conjugated with fluorescein isocyanate. The details of the technic employed for the preparation and purification of fluorescent conjugates were similar to those previously described.[6,7] Bone marrow aspirates and peripheral blood were obtained from three normal patients, one patient with polycythemia vera and five patients with idiopathic thrombocytopenic purpura (ITP). Thin bone marrow smears were prepared, air dried, stored in the refrigerator (5 C.) and immunochemically stained within 24 hours. Platelets were obtained by differential centrifugation of heparinized blood; smears were made and treated in a similar fashion to the bone marrow smears. All smears were made on chemically clean glass slides; no further treatment of glassware was found necessary to preserve these cells for immunochemical stains.

Splenic tissue was obtained at autopsy from three "normal" (non-thrombocytopenic) cases. Spleens were obtained at surgery in two cases of idiopathic thrombocytopenic purpura. The processing of tissues and smears and the immunochemical staining technic was similar to that previously described.[6,8]

The tissue sections and smears were stained with fluorescent antiplatelet conjugates for 45 minutes. Controls for the specificity of the results consisted of staining similar sections and smears with fluorescent anti-human fibrinogen; anti-human gamma globulin; anti-human albumin and heterologous fluorescent conjugates (anti-rabbit fibrinogen, gamma globulin and albumin). The methods for obtaining these different conjugates have already been described.[6,9] Other technical controls consisted of applying nonfluorescent antiplatelet serum to the slides prior to the staining with fluorescent anti-platelet conjugate, in order to block immunologically the specific fluorescence. Likewise, as controls, absorption of the fluorescent anti-platelet conjugate with human platelets as well as with fibrin were used prior to the immunochemical staining. Other technical controls as well as the optical system and photographic technics employed were similar to those previously reported. Parallel observations of a given field were done with both fluorescence and phase microscopy for the morphologic and immunochemical correlation of the cells under study. Comparable smears and tissues were also routinely stained with Wright's stain.

Results

Platelets

When stained with fluorescent anti-platelet conjugate, platelets showed bright green specific fluorescence (fig. 1) that under the conditions of our experiments appeared to involve uniformly the whole body of the cell. An occasional platelet smear that was allowed to remain air dried for long periods (weeks or longer) showed the specific fluorescence suggestively more intense at the periphery of the cells than in the central portions. All of the control reactions were similar to those to be described for megakaryocytes.

Bone Marrow

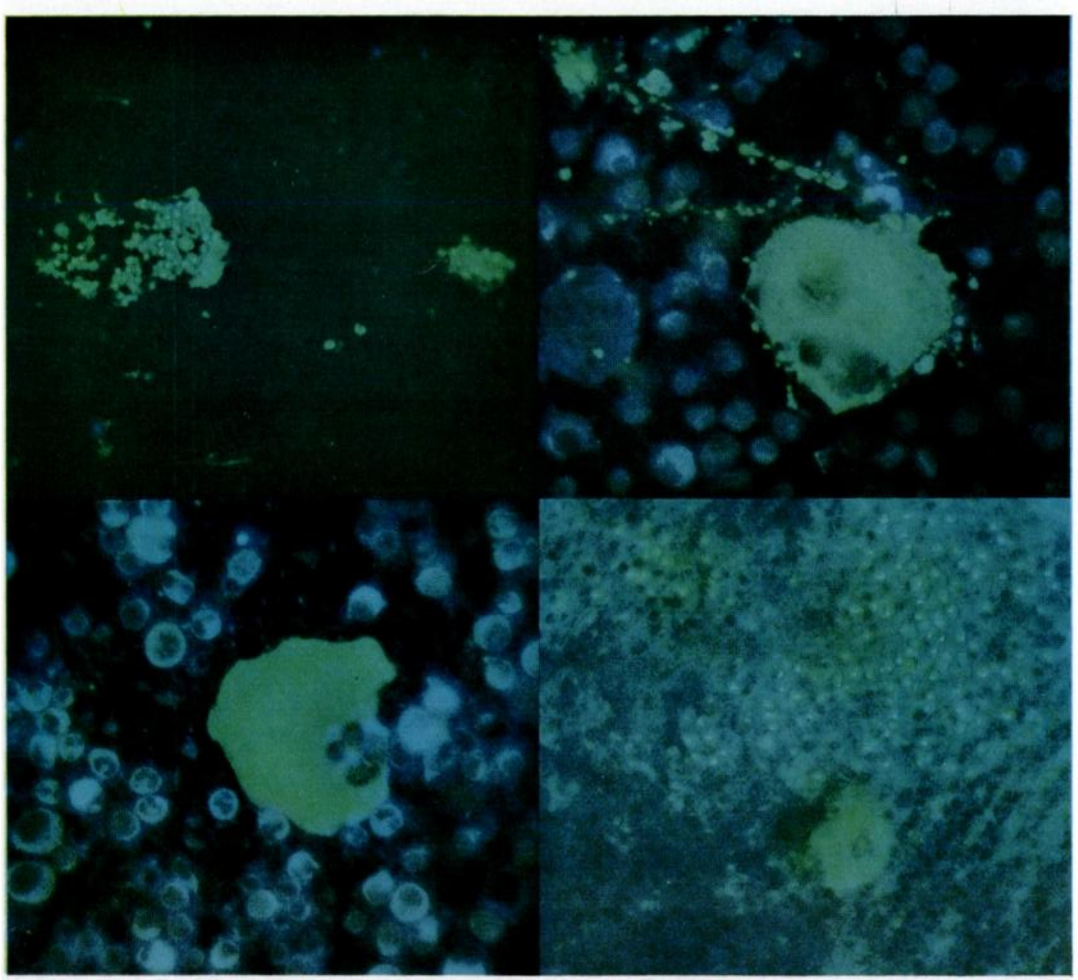
When bone marrow smears of normal and polycythemic cases were stained with the fluorescent anti-human platelet conjugate, specific green fluorescence was observed in the cytoplasm of megakaryocytes. Most megakaryocytes were of the platelet-producing type and the fluorescence assumed a granular character. Usually the multiform nuclei stood out due to their lack of

FIG. 1 *(top, left).*—Smear of normal human platelets obtained by differential centrifugation of heparinized blood. Note the bright green specific fluorescence of clumps of platelets.

FIG. 2 *(top, right).*—Smear of normal human bone marrow showing a platelet-producing megakaryocyte. Note the bright green specific fluorescence of a granular character in the cytoplasm of the cell. The nuclei stand out by their lack of fluorescence. Also note specifically stained platelets at the periphery of the cell. Some of them appear caught in a fibrin thread. The fibrin does not stain with the fluorescent conjugate.

FIG. 3 *(bottom, left).*—Bone marrow smear from a case of ITP showing a nonplatelet-producing megakaryocyte. Note by contrast with figure 2 the homogeneous character of the specific cytoplasmic fluorescence in this cell.

FIG. 4 *(bottom, right).*—Section of spleen from a case of ITP. Note the large number of bright green specifically stained platelets in the red pulp. A megakaryocyte of suggestively homogeneous character is also seen.



Figs. 1–4.—Fluorescent micrographs of human cells and tissues stained with fluorescent anti-human platelet conjugate.

fluorescence. Formed platelets with specific fluorescence could be seen in the periphery of these cells as well as budding from them (fig. 2). Rare nonplatelet-producing megakaryocytes showed also specific fluorescence but of a diffuse homogeneous character. In many instances, streams of platelets along fibrin threads could be seen in the bone marrow preparations, the platelets staining specifically although the fibrin threads were not stained. Cells other than megakaryocytes and platelets lacked specific fluorescence and showed in most preparations blue autofluorescence. In an occasional instance, particularly in smears not washed prior to immunologic fixation, polymorphonuclear leukocytes showed "nonspecific" green fluorescence as previously described.[8] Such staining could be reduced although not completely removed by adsorption of the fluorescent conjugate with bone marrow powder.

Bone marrow preparations from cases of ITP (fig. 3) showed a similar specific fluorescence in the megakaryocytes. The majority of these cells had a homogeneous fluorescence and peripheral platelets were not observed. The total number of these nonplatelet producing megakaryocytes appeared greatly increased.

When similar smears were stained with a fluorescent anti-platelet conjugate that had been previously absorbed with human fibrin no appreciable difference in the specific staining of megakaryocytes was observed. On the other hand, absorption of the fluorescent conjugate with packed platelets greatly diminished the specific fluorescence of the cells in smears. When the bone marrow smears were stained with fluorescent anti-human fibrinogen (or fibrin) specific green fluorescence was seen in many megakaryocytes although of much less intensity than that observed with fluorescent anti-platelet conjugate; on the other hand, fibrin threads stained with bright specific fluorescence. The mild specific staining of megakaryocytes by fluorescent anti-human fibrinogen could be greatly diminished or abolished when the fluorescent anti-human fibrinogen was absorbed with fibrin prior to staining. No specific fluorescence in megakaryocytes was observed when the bone marrow smears were stained with fluorescent anti-human gamma globulin or anti-human albumin; only extracellular areas of these proteins (plasma) were occasionally stained in these instances, mainly in unwashed smears. Similarly, no specific fluorescence was observed when smears were stained with fluorescent antibody conjugates to heterologous proteins.

Spleen

When spleen sections of nonthrombocytopenic cases were stained with anti-human platelet conjugates, an occasional small clump of platelets or isolated platelets could be seen scattered throughout the red pulp. Seldom could a megakaryocyte be identified in these preparations. In either case, platelets and megakaryocytes stood out brilliantly by virtue of their specific green fluorescence. Again, this observation was controlled with similar results to those found in bone marrow. It was difficult to ascertain whether the platelets were in sinusoids or in the intersinusoidal areas in the spleen. When

sections of the spleen in two cases of ITP were stained with fluorescent anti-human platelet conjugates, the platelets and megakaryocytes stained brightly, as in normal spleens (fig. 4), but the number of platelets in these spleens was greatly increased (five or more times). They were scattered throughout the red pulp and as a rule were absent from the follicles. It could not be ascertained whether the platelets were located in the lumen of sinusoids or whether they were "trapped" in the intersinusoidal area. Cells other than platelets and megakaryocytes in the spleen lacked specific fluorescence when stained with the fluorescent anti-platelet conjugates. All the control reactions were similar to those described under bone marrow.

Discussion

The results of our study add direct evidence to support the hypothesis that platelets are derived from megakaryocytes. As it is not well known whether platelets per se possess a single or complex antigenic structure, one can only conclude that at least one common antigen is present in platelets and in the cytoplasm of megakaryocytes, both in nonthrombocytopenic cases and in cases with idiopathic thrombocytopenic purpura. Whether megakaryocytes in the normal and in idiopathic thrombocytopenia have the same antigenic structure, or whether in the latter condition some antigens are lacking or added in these cells cannot be established by our studies. The lack of specific fluorescence in nuclei of megakaryocytes is given as suggestive evidence against the belief that the nucleus of these cells also contributes to the formation of platelets.[10,11]

The work of Seligmann et al.[12] establishing the presence of a substance with the antigenic specificity of fibrinogen in extracts of repeatedly washed platelets is supported by our findings of slight specific fluorescence of platelets (and megakaryocytes) when stained with fluorescent antifibrinogen. Whether this material is forming part of the body of these cells or whether it is adsorbed on the surface cannot be ascertained from this study. On the other hand, fibrin threads were not specifically stained with the fluorescent anti-platelet conjugate. This indicates that if any fibrinogen-like material was present in the platelets used as antigen in these experiments, it did not elicit an antibody response detectable by these immunohistochemical procedures.

The megakaryocytes from the bone marrow of the patients suffering from idiopathic thrombocytopenia could be differentiated with ease from most normal megakaryocytes. The cytoplasm had a homogeneous fluorescence appearing "waxy" with little or no sign of granularity or platelet production. These observations confirm the findings of Dameshek and Miller,[13] who employed conventional staining technics.

The finding of a marked increase in the number of platelets in the spleens of cases with idiopathic thrombocytopenic purpura might support the concept of sequestration of platelets in the pathogenesis of this disease. However, one must be cautious in comparing the number of platelets found in surgically removed spleens with those found in autopsy material. It is possible that

factors such as surgical trauma, manipulation, etc., might play a role in at least partially increasing the number of these cells in the spleen, such an increase having no pathogenetic significance in the thrombocytopenia seen in these cases.

It is felt that the fluorescent antibody technic of Coons et al. is a valuable tool for the immunochemical and morphologic study of platelets and megakaryocytes, both in the normal and in cases of hematologic disorders. This is particularly true when structure and localization of these cells are sought in tissues, as this method is perhaps the best available at the present for this purpose.

Summary

By means of the fluorescent antibody technic of Coons and Kaplan it was possible to demonstrate a common antigenic structure in human platelets and megakaryocytes, both in nonthrombocytopenic cases and in cases with idiopathic thrombocytopenic purpura. Direct evidence for a marked increase in the number of platelets in the spleens of two cases of idiopathic thrombocytopenia is given. The pathogenetic significance of this finding is discussed. It is concluded that the fluorescent antibody technic is a valuable tool for the chemical and morphologic study of platelets and megakaryocytes both in tissues and smears.

Summario in Interlingua

Per medio del technica de Coons e Kaplan a anticorpore fluorescente il esseva possible demonstrar le presentia de un commun structura antigenic in plachettas e megacaryocytos human, tanto in casos nonthrombocytopenic como etiam in casos de idiopathic purpura thrombocytopenic. Evidentia directe es presentate de un marcate augmento in le numero de plachettas in le splen in duo casos de thrombocytopenia idopathic. Le signification pathogenetic de iste constatation es discutite. Es concludite que le technica a anticorpore fluorescente es un instrumento de valor pro le studio chimic e morphologic de plachettas e megacaryocytos in tissus e in frottis.

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INHIBITION OF HEPARIN CLEARING BY PLATELETS. *J. R. A. Mitchell.* From the Department of the Regius Professor of Medicine, The Radcliffe Infirmary, Oxford, England. Lancet *1*:169, 1959.

Platelet-rich lipemic plasma cleared less rapidly and completely than platelet-poor plasma, following the addition of plasma obtained 5 minutes after an intravenous injection of heparin. A cell-free, heat-stable, non-dialysable extract of platelets was prepared, which inhibited the clearing of platelet-poor lipemic plasma and of fat emulsions by post-heparin plasma and by pancreatic lipase.—*R. M. H.*