The Application of Fluorescent Antibody Methods to the Study of Platelets

By ROBERT SIUKE, ROBERTO BENITEZ, WARREN C. EVELAND, JOSEPH H. AKEROYD and CHARLES J. DUNNE

CHEMICAL LINKAGE of fluorescent dyes with antibody allows histologic detection of antigen-antibody reactions. This technic, originally developed by Coons, has been extensively employed to localize the antigens of and antibodies to such diverse substances as bacterial products, viruses, blood group substances, hormones and many proteins of normal or abnormal nature.

Platelet antibodies occur in humans and are readily prepared in animals; thus, through fluorescent antibody technic, an immunohistologic approach to platelet antibody-antigen systems, as well as to their in vivo sequestration sites seemed possible. Humphrey and Craig and Gitlin have carried out investigations in this field. The purpose of this report is to present our observations on the immunocytologic characteristics of platelets employing the Coons technic. Similar studies have been carried out independently at the University of Pittsburgh School of Medicine and are being reported elsewhere in this issue.

MATERIALS AND METHODS

Preparation of platelet antiserum.—Siliconized glassware was used in preparing the platelet concentrates. Platelets were obtained from two donors of different blood groups (J. E., O Rh positive; J. O., B Rh positive), using one volume of one per cent disodium ethylenediaminetetra-acetic acid (EDTA) in saline as anticoagulant for nine volumes of blood.

The blood was centrifuged immediately after collection at 28 × g for 25 minutes in a Servall refrigerated centrifuge to yield platelet-rich plasma. The platelets were washed three times with one per cent EDTA in saline and resuspended in 0.9 per cent saline to one-tenth of the platelet rich plasma volume. Five-tenth ml. of this suspension (containing approximately one billion platelets) with S. typhosa lipopolysaccharide* as adjuvant at a 5 μg./ml. concentration was injected intravenously into chinchilla rabbits at three day intervals for three injections. Thereafter, the animals were injected intraperitoneally at weekly intervals for four more injections. The rabbits were bled one week after the last intraperitoneal injection. The serum was inactivated in a water bath at 56 C. for 30 minutes, absorbed several times with washed human red blood cells and stored at —20 C. This method usually produced antisera of adequate titer; the characteristics of a typical working serum are shown in table 1.

Antiserum against dog platelets was similarly prepared using pooled dog platelets.

Rabbit antihuman fibrinogen serum was kindly supplied by Hyland Laboratories where it had been prepared with fibrinogen purified from Cohn Fraction I containing 80 per

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*Prepared and supplied by Dr. Maurice Landy.
Table 1.—Rabbit Anti-Human Platelet Serum 1823.

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Platelet agglutination</th>
<th>Specific fluorescence</th>
<th>Indirect method</th>
<th>Inhibition of clot retraction</th>
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</thead>
<tbody>
<tr>
<td>1:1</td>
<td>4+</td>
<td>4+</td>
<td>Complete</td>
<td></td>
</tr>
<tr>
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<td>4+</td>
<td>4+</td>
<td>Complete</td>
<td></td>
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<tr>
<td>1:4</td>
<td>4+</td>
<td>4+</td>
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<td></td>
</tr>
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<td>3+</td>
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<td>1+</td>
<td>0</td>
<td>3+</td>
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<tr>
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<tr>
<td>1:1024</td>
<td>0</td>
<td>0</td>
<td>±</td>
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Table 2.—Results of Treatment of Platelet Smears with Fluorescent Antibody

<table>
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<th>Direct method</th>
<th>Specific fluorescence</th>
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<tbody>
<tr>
<td>Unstained slide</td>
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</tr>
<tr>
<td>Fluorescent rabbit anti-human platelet serum</td>
<td>3+</td>
</tr>
<tr>
<td>Fluorescent normal rabbit serum</td>
<td>0</td>
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<tr>
<td>Fluorescent Coombs' serum</td>
<td>0</td>
</tr>
<tr>
<td>Indirect method</td>
<td></td>
</tr>
<tr>
<td>Rabbit anti-human platelet serum followed by fluorescent sheep or goat anti-rabbit serum</td>
<td>4+</td>
</tr>
<tr>
<td>Normal rabbit serum followed by fluorescent sheep or goat anti-rabbit serum</td>
<td>0</td>
</tr>
<tr>
<td>Absorption</td>
<td></td>
</tr>
<tr>
<td>Fluorescent rabbit anti-human platelet serum after absorption with platelets</td>
<td>0</td>
</tr>
<tr>
<td>Blocking</td>
<td></td>
</tr>
<tr>
<td>Fluorescent rabbit anti-human platelet serum blocked by non-fluorescent rabbit anti-human platelet serum</td>
<td>1+</td>
</tr>
</tbody>
</table>

Preparation of platelet material.—Platelet smears were prepared by placing a drop of a dilute suspension of thrice washed platelets on scrupulously clean slides, which were allowed to air-dry and then fixed in methyl alcohol for 10 minutes. The fixed slides were stored at -20 C. for periods of time up to six months with no apparent effect on the staining reaction with fluorescent antibody.

Preparation of tissues.—Clots were prepared in vitro by recalcification of the following materials which had been kept in EDTA-saline anticoagulant: blood, platelet-rich plasma (containing approximately 400,000 platelets per cubic milliliter), platelet-rich plasma containing 20 volumes per cent erythrocytes, platelet-rich plasma with 2 volumes per cent erythrocytes, platelet-concentrated plasma with a platelet concentration of 2,000,000/cu.mm., and platelet-free plasma clots.

*Nutritional Biochemical Company, Cleveland, Ohio.
For histologic sections, the clots were quick-frozen at $-70\,^\circ\mathrm{C.}$ in test tubes dipped in a mixture of dry ice and alcohol. They were sectioned in a cryostat at $-15\,^\circ\mathrm{C.}$, and fixed in methyl alcohol before use. Splenic tissue was obtained at autopsy or at the time of splenectomy. Small pieces were frozen in a test tube in liquid nitrogen ($-195\,^\circ\mathrm{C.}$), and sectioned with the cryostat. Slides were fixed in ether-alcohol, as suggested by Vazquez and Dixon. Bone marrow coverslip preparations were fixed in the same manner. This method of fixation was generally superior to methyl alcohol fixation.

"Staining" was done by the direct and indirect technics in all experiments. In the direct method fluorescein conjugated serum was applied to the preparation for thirty minutes, then rinsed off with 0.05 M phosphate buffer and finally washed in buffer under constant agitation for 10 minutes. Buffered glycerol was used as mounting medium. In the indirect method nonconjugated serum was applied to the slide for 30 minutes, rinsed and washed off as above. This was followed by a second stage consisting of the application of a fluorescein conjugated serum containing antibody against the species antiserum used in the first stage. Usually the first stage was either rabbit or human serum followed by goat or sheep anti-rabbit or rabbit anti-human fluorescent serum, respectively. The second stage antiserum was applied for 30 minutes, the slides were rinsed, washed and mounted as already described.

The preparations were examined through a Leitz Ortholux microscope. Fluorescence was excited by a Phillips 150 watt mercury arc lamp. UG 1 or BC-12 filters were generally used with a secondary Wratten 2A or OG-4 filter.

**RESULTS**

**Heterospecific Antiserum**

When fluorescent rabbit anti-human platelet serum is applied to washed human platelets, a halo of green fluorescence is seen around each platelet (fig. 1). This did not occur when platelets were treated with fluorescent normal rabbit, sheep anti-rabbit or rabbit Coombs serum, by either the direct or the indirect technics. Absorption of fluorescent rabbit anti-human platelet serum with human platelets abolished all specific fluorescence, whereas absorption with human erythrocytes or guinea pig tissue powder failed to do so. "Blocking" by treatment of the smears with nonconjugated rabbit anti-human platelet serum either before or simultaneously with fluorescent rabbit anti-human platelet serum appreciably diminished but did not fully abolish fluorescence.

Species specificity was investigated with slides of human platelets, dog platelets and guinea pig platelets. Fluorescence was found when antisera against platelets of one species were applied to platelets of another species; however, the intensity of fluorescence was less than when the reaction was species specific.

The validity of a strict comparison between the sensitivity of other methods and the Coons technic in detecting heteroantibody is questionable since only very limited quantitative interpretation can be ascribed to the data obtained. In general, fluorescent antibody indirect technic was as sensitive as the agglutination method and somewhat more sensitive than the direct technic. As could be expected, clot retraction inhibition was the least sensitive method tested (table 1).

The degree of fluorescence was not appreciably affected by variations in temperature from 4 to 37 $^\circ\mathrm{C.}$ during the reaction between human platelets
and rabbit anti-human platelet fluorescent serum. No increase in the intensity of specific fluorescence was obtained by lengthening the reaction time beyond 30 minutes. Fixation in 10 per cent formalin for 24 hours greatly diminished fluorescence. This observation discouraged us from using formalin-fixed tissue in these studies. Rabbit anti-human platelet serum reacted with A, B and O platelets regardless of the platelets' blood group used to immunize the rabbits.

Fluorescent rabbit anti-human platelet serum readily demonstrated platelets within the artificially made clots. Groups of platelets were usually enmeshed in the interstices of the fibrin network. Absorption of the antiserum with an equal volume of packed platelets abolished fluorescence of platelets in tissue, while absorption with an equal volume of erythrocytes or fibrin failed to inhibit the reaction. When platelet concentration in clots was kept constant and the concentration of erythrocytes was varied, the degree of platelet fluorescence varied inversely with the erythrocyte concentration. This observation is compatible with the quenching of fluorescence by erythrocytes previously noted in other systems. It has been suggested that this effect may

Fig. 1.—Human platelets stained with heteroantibody (indirect technic).
be caused by hemoglobin.\textsuperscript{10} Fluorescent normal rabbit serum did not cause fluorescence of platelets or fibrin in the clots.

**Blood Group Antigens**

Attempts to demonstrate the A, B and D antigens in platelets were repeatedly unsuccessful. Treatment of platelet slides from A Rh positive and B Rh positive donors with anti-A, anti-B and anti-D serum was carried out at 4, 23 and 37 C., for time intervals ranging from 30 minutes to 12 hours. No specific fluorescence was seen by either the direct or the indirect technic in these or control slides despite the use of several high titer immune sera. The "layering" technic, which consists of adding three or more stages with each subsequent anti-serum directed at the serum of the previous stage, was also unsuccessful. The demonstration of A and B antigens in platelets with fluorescent antibody using unfixed platelets has recently been reported.\textsuperscript{11} Our attempts to confirm these findings using platelet suspensions met with equivocal results due to non-specific staining.

**Platelet \textquotedblleft Plasmatic Atmosphere\textquotedblright\textsuperscript{12}**

The coating of platelets with plasma proteins was studied with fluorescent rabbit Coombs and rabbit anti-human fibrinogen sera. After one or two washings with EDTA in saline the fluorescent Coombs serum revealed proteins still associated with the platelets. However, a third washing was usually sufficient to result in a negative reaction between platelets and the fluorescent Coombs serum. This suggests that if any serum protein is still adhering to the platelets after three washings, it is present in insufficient quantity to be detectable by this technic.

On the other hand, even after platelets had had as many as 10 washings in EDTA-saline, treatment with antifibrinogen serum always resulted in specific fluorescence, thus indicating that fibrinogen had not been removed. Absorption of the antifibrinogen serum with 30 mg./ml. of human fibrinogen abolished its reaction with platelets, while absorption with 30 mg./ml. of Cohn fraction IV did not inhibit the reaction. These observations, which confirm the association of fibrinogen with platelets reported by Seligmann et al.,\textsuperscript{13} do not clarify whether fibrinogen is part of the platelet structure or just firmly absorbed onto it.

**Idiopathic Thrombocytopenic Purpura**

Nineteen sera from patients suffering with idiopathic thrombocytopenic purpura (ITP) were studied; eight of these sera had been supplied by Dr. W. J. Harrington, who had found six of them to give positive reactions for platelet agglutinins when tested by his method.\textsuperscript{14} All sera were tested against platelet slides from A, B and O donors by the indirect technic using fluorescent rabbit Coombs' serum for the second stage. Nine of the ITP sera were conjugated with fluorescein isothiocyanate as were an equal number of control sera. In these experiments, as in the blood group studies, sera were applied to the slides for time periods ranging from 30 minutes to 12 hours, and over a temperature range of 4 to 37 C. In one experiment the platelet slides had been
treated with ficin prior to treatment with the ITP sera. In no case was specific fluorescence of platelets noted.

Tissue Studies

Bone marrow aspirates were stained with conjugated anti-human platelet serum. All megakaryocytes showed bright fluorescence which was not present when normal rabbit, sheep anti-rabbit or rabbit Coombs serum were applied to the preparations, although some nonspecific leukocyte fluorescence was encountered with all fluorescent sera (fig. 2).

Four spleens obtained from hematologically normal patients at autopsy and two spleens obtained at splenectomy from patients with idiopathic thrombocytopenic purpura were 'stained' for platelets. Scattered within the splenic cords bright green fluorescent platelets were seen singly and in clumps of variable size. Identity as platelets was confirmed by using control sera which produced no specific staining. When antiserum was absorbed with packed thrombocytes no specific fluorescence was observed. The idiopathic thrombocytopenic purpura spleens contained platelets in great abundance. In fact, platelets were numerically greater on sections from ITP spleens than from the

Fig. 2.—Human megakaryocyte stained with heteroantibody (direct technic).
spleens of patients with nonhematologic disease. However, comparison of autopsy spleens with surgical material is not valid and no quantitative conclusions can be drawn. The indirect technic was unsatisfactory in tissue studies because of its appreciable background fluorescence.

**DISCUSSION**

Fluorescent platelet antiserum was first used by Humphrey in the guinea pig to demonstrate the antigenic kinship between homologous platelets and megakaryocytes. He interpreted the reaction in anti-platelet serum with megakaryocytes as evidence that the latter is the progenitor of platelets; we have confirmed his observation. Craig and Gitlin found no specific fluorescence when platelet antisera were applied to the lesions of thrombotic thrombocytopenic purpura. Although the thrombi failed to react with fluorescein-labeled rabbit anti-human platelet serum, they did react with fluorescent anti-human fibrin antibody. On the basis of these observations the authors concluded that the hyaline thrombi in thrombotic thrombocytopenic purpura are composed of
a derivative of fibrinogen or fibrin and that platelets were not present in them. They do not give many details concerning the anti-platelet serum in their report nor do they demonstrate that this antiserum was actually able to detect platelets in clots or tissues. This information would be desirable to substantiate the negative evidence obtained with the platelet antiserum; furthermore, our finding of a strong reaction between antifibrinogen serum and platelets shows that fluorescence following treatment of thrombi with a fibrin antibody is of questionable significance in deciding whether platelets are present or absent in a clot because of the intimate association of fibrinogen with platelets. In this light, we cannot interpret their data as proof of the absence of platelets from the thrombi of thrombotic thrombocytopenic purpura.

Our studies indicate that the reaction between platelets and fluorescent heteroantibody can be detected in a reproducible, specific and sensitive fashion. The method was then applied to study the serum of normal individuals and of patients with idiopathic thrombocytopenic purpura. Although there is considerable evidence suggesting that this is a disease of autoimmunization, we have been unable to demonstrate an antibody by the fluorescent technic in ITP sera known to have an appreciable titer of platelet agglutinating factor.

The striking difference found between the behavior of heterospecific antibody and the platelet agglutinating factor of ITP is akin to the experience of Coombs, who was unable to detect platelet antibody in ITP sera by the mixed erythrocyte-platelet anti-globulin reaction while being able to detect platelet antibody in rabbit serum. In addition to the possibility that this difference may reflect a greater number of available antigenic sites in the platelet for heteroantibody than for homologous antibody, Coombs has suggested two other explanations for this discrepancy. The thorough washing necessary in both technics could remove an antibody which coats platelets. Easy dissociation of a platelet-antibody complex was shown by Shulman in quinidine purpura. Negative results would follow fluorescent and mixed agglutination methods if the ITP antibody dissociated readily, while agglutination technics, in which platelets are not washed after exposure to serum, would reveal the presence of antibody. It is likely then, that if the platelet agglutinating factor in ITP sera is an antibody its characteristic behavior toward the platelet antigen differs from heterospecific antibody.

The other possibility suggested by Coombs is that the platelet agglutinating factor may not be a true antibody. If this were the case, we could not expect to visualize it by the fluorescent technic. Since the negative results obtained with ITP sera containing platelet agglutinating factor are compatible with either explanation no conclusion on the immunologic vs. nonimmunologic character of platelet agglutinating factor can be drawn from these findings.

Specific fluorescence shown only by megakaryocytes and platelets following application of anti-human platelet serum to tissue can be attributed to the presence of antigens in platelets which are absent from other cells. It remains exceedingly difficult to recognize platelets in tissue despite various methods found in the older literature. The immunochemical approach with fluores-
cent antibody permits clear-cut histologic localization of platelets in splenic tissue and offers another tool in the study of platelet sequestration sites.

**Summary**

1. Through the use of fluorescent antibody technic, it is possible to detect the antigen-antibody reaction between platelets and heterospecific antiserum, but no antigen-antibody reaction between normal human platelets and sera from patients with idiopathic thrombocytopenic purpura could be demonstrated.

2. The method permits the histologic localization of platelets in splenic tissue.

3. Whereas serum proteins can be easily removed from platelets by washing, there is an intimate association with fibrinogen so that this protein is still not removed from the platelet after 10 washings in saline.

**Summary in Interlingua**

1. Per medio del technica a anticorpore fluorescente il es possibile detegere le reaction antigeno-anticorporee inter placchettas e antisero heterospecific, sed nulle tal reaction poteva esser demonstrate inter normal placchettas human e seros ab patientes con idiopathic purpura thrombocytopenic.

2. Le metodo permette le localisation histologic de placchettas in tissu splenic.

3. Durante que proteinas seral pote esser removite facilmente ab le placchettas per lavar los, il existe un association intime con fibrinogeno, de maniera que iste proteina es non ancora removite ab le placchettas post 10 lavationes in solution salin.

**References**


FLUORESCENT ANTIBODY METHODS AND STUDY OF PLATELETS


In many neoplastic tissues the mean amount of DNA per cell is greater than that in normal tissues. Therefore, if cells of normal appearance in cases of leukemia have a DNA content different from the DNA content of similar cells in normal individuals, then the determination of DNA in the cells of leukemic blood may be useful in early diagnosis. Recent advances in technic permit accurate measurement of the amount of absorbing material in objects of irregular outline and with uneven distribution of absorbing material. These measurements were made on an integrating micro-densitometer after Feulgen staining. Blood was drawn from 5 men and 5 women on two occasions, and 15 cells were examined in each sample. All normal samples showed a narrow distribution of the amount of absorbing material present in the cells. Abnormal samples were obtained from 15 cases of leukemia and related conditions. In all cases of leukemia the leukocytes showed a much wider distribution of absorbing material. In the majority the distribution shows that the variation extends from the lower normal limit to approximately double that value. If there are only a few circulating cells with high values, the mean value for the DNA content of leukocytes is close to the normal modal value. On the other hand, if there is a large number of primitive cells present, the mean value may be up to approximately 30 per cent greater than normal. Micro-photometry is of little value in trying to identify abnormal cells in leukemia. If the cell is abnormal to circulating blood, then it will be obvious on inspection.—O. P. J.
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