## Fluorescent Dye Uptake By Platelets.* (32585)

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Sokal(1) reported that auramine O stained platelet granules orange and cytoplasm green when viewed under fluorescent light. He noted that the granules disappeared when platelets were aggregated by thrombin but observed no loss with ADP aggregation.

In the present study, a similar fluorescent staining method was used to investigate reactions of platelet granules in the presence of platelet antibody and under various other conditions.

*Materials and methods.* Platelet rich plasma (PRP) samples were obtained by plasmapheresis from donors at The University of Texas M. D. Anderson Hospital and Tumor Institute, 500 ml of blood being collected in plastic bags containing 75 ml ACD-A solution. The PRP was tested within 2 hours after being shed from the donor and had a pH ranging from 7.0 to 7.2. As a normal control, 0.9 ml PRP was incubated with 0.1 ml of euchrysine in aqueous or saline solution at a dilution of 1:5000 for 1 hour at 37°C. A drop was placed on a slide and the preparation was observed under oil immersion with a Zeiss fluorescent microscope equipped with an Osram HBO 200 watt lamp and a dark-field condenser. A BG 12 exciter filter and a 50/44 barrier filter combination were used.

Rabbit antiplatelet serum was prepared by 3 biweekly intravenous injections of approximately 1 billion washed human platelets. Serum was collected 2 weeks after the last injection. The antiserum was absorbed with washed human type A and type B red blood cells and with lyophilized human serum. By the indirect fluorescent antibody technique using methanol-fixed washed platelets as the antigen, the titer was 1:160.

Reagents and the sources and concentrations used are shown in Table I.

*Results.* As reported by Sokal(1), platelets incubated with euchrysine in either aqueous or isotonic saline at 37°C showed orange granules and green cytoplasm when examined by fluorescent microscopy. When normal rabbit or normal human serum was added to the preparation at a dilution of 1:5 or greater, the granules were likewise prominent. When rabbit antihuman platelet serum or the gamma globulin fraction of such serum was used in place of normal serum, granules were not visible. Also platelets were round and failed to spread normally on glass in the presence of platelet antibody. This effect was obtained with dilutions of antisera up to 1:400, and it could be reduced by absorption of the antisera with washed platelets. The failure to stain granules could be obtained with rabbit antiserum to human red cell stroma, and again it could be reduced by absorption of the serum with red cell stroma.

When commercially available anti-A typing serum was used with platelets from a type A individual, dye uptake by platelet granules was not affected. Likewise, sera from patients who had received multiple platelet transfusions and who showed rapid loss of transfused platelets from the circulation did not have a demonstrable effect on dye uptake although there was agglutination in low dilutions by sera from 2 of 10 patients tested. Platelets of a type O individual stained normally when incubated with A and B blood group substances. Endotoxin of the Boivin type in concentrations of 50, 100, and 500 $\mu$g/ml and streptolysin O (reconstituted in distilled water and used in the proportion of 1 part in 5 of the PRP-dye mixture) were also without effect.

When 1 unit of bovine thrombin/ml PRP was added to the PRP-dye mixture, orange granules were not visible after incubation, and the round platelets resembled those which were exposed to rabbit anti-platelet serum. Higher concentrations of thrombin caused the formation of large clots and obscured the individual platelets. Addition of 1 mg of

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TABLE I. Reagents Used in Studies of Fluorescent Dye Uptake by Platelets.

| Reagent | Diluent | Concentration/ml PRP | Source |
|---|---|---|---|
| Euchrysine | Water<br>Saline | 1:50,000 | George T. Gurr, Ltd. |
| Thrombin | Saline | 2, 1, 0.5, 0.25, 0.125 U | Parke, Davis and Co. |
| Adenine | Water<br>ACD | 1 mmol, 0.5 mmol | Matheson, Coleman and Bell |
| Adenosine diphosphate (ADP) | Buffered saline pH 7.2 | 10 $\mu$g | K and K Laboratories |
| Adenosine triphosphate (ATP) | " | " | " |
| Adenosine monophosphate (AMP) | " | " | " |
| Adenosine | " | " | " |
| Endotoxin: | | | |
| *E. coli* 055: B 5 | Saline | 500, 100, 50 $\mu$g | Difco Laboratories |
| *E. coli* 026: B 6 | " | " | " |
| *S. typhosa* 0901 | " | " | " |
| Cortisone acetate | Saline | 5, 2½, 1, 0.5, 0.25, 0.1 mg | Schering |
| Heparin | Saline | 1, 0.5, 0.25, 0.125, 0.06 mg | Riker Laboratories |
| A and B blood group substances | Saline | 0.1, 0.05, 0.01, 0.001 ml | Knickerbocker Biologicals |
| Anti A and anti B sera | Saline | 1:5, 1:10, 1:20 | Spectra Biologicals |
| Adrenaline chloride | Saline | 1:5000, 1:10,000, 1:100,000 | Parke, Davis and Co. |
| Streptolysin O | Water | 1:5 of reconstituted compound | Hyland Laboratories |

heparin to the platelet-antibody preparation did not alter the results, although the same amount did abolish the degranulation by 1 unit/ml of thrombin.

Other conditions which altered the uptake of dye by platelets were incubation of the PRP-dye mixture at 1°C or reduction of the pH to 6.5. Under these conditions, the granules remained unstained until the preparation was warmed (27°C to 37°C) or the pH was raised to 7.0, when they became orange.

Table II shows that platelet aggregating agents such as ADP and adrenaline, as well as inhibitors such as ATP and AMP had no effect on dye uptake. Cortisone up to 1 mg/ml did not influence dye uptake by platelets in the presence of antibody whether it was added to the PRP before or after antisera.

When PRP was stored at 5°C for periods up to 10 days, granules retained the capacity to take up dye. If storage was at an acid pH of 6.5, dye uptake was normal when platelets were resuspended at pH 7.0.

Granules were stained during the early stages of normal spreading on glass but were not demonstrable when the outline of the

TABLE II. Effect of Various Treatments on Fluorescent Dye Uptake by Platelet Granules.

| Treatments causing failure of uptake | Treatments having no effect on uptake |
|---|---|
| 1. Rabbit anti-platelet, serum, to 1:400 | 1. ADP, AMP, adenosine, ATP and adenine —10 $\mu$g/ml |
| 2. Rabbit anti-stroma serum to 1:200 | 2. Endotoxin, 500 $\mu$g/ml |
| 3. Incubation @ 1°C, reversible on warming | 3. Storage 10 days @ 5°C |
| 4. Incubation @ acid pH, reversible @ pH 7.0 | 4. Normal human or rabbit serum 1:5 |
| 5. Thrombin, 1 unit | 5. Anti-A serum, platelets from type A blood |
| | 6. Streptolysin O, 1:5 reconstituted dilution in PRP |
| | 7. Adrenaline, 1:5,000 |
| | 8. Blood group specific substances A and B with platelets from type O blood |
| | 9. Cortisone, 1 mg/ml |

platelet no longer was visible.

*Discussion.* In the present study, exposure to thrombin and to platelet or red cell stromal antibody had the greatest effect on dye uptake by platelet granules. With these, granules were not visible after the incubation period. Although the reactions of platelets with antisera were morphologically similar to those with thrombin, as was also noted by Balboa *et al* (2), the antisera effects apparently could not be caused by residual thrombin since normal rabbit or human serum, which presumably would contain similar amounts of thrombin, permitted the usual uptake of dye. Furthermore, the gamma globulin fraction of antiplatelet serum was as effective as whole serum in the inhibition of staining, and neutralizing any residual thrombin by adding heparin to antiplatelet serum did not alter these results.

Possibly, antistromal serum reacted in a manner similar to antiplatelet serum since platelets and red cells are believed to share some common antigens(3,4); however, anti-A and anti-B were without effect. Also the presence of an antigen-antibody complex such as that of platelets in type O plasma with added soluble A and B blood substances was without effect.

Marcus *et al*(5) believe that platelet granules have a composition compatible with that of lysosomes. Acid phosphatase activity has been associated with the granules of platelets, and such activity has been used as a marker of lysosomes by De Duve(6). Allison and Mallucci(7) found that the lysosomes of leukocytes showed orange fluorescence. If the orange granules in platelets are lysosomes, they did not seem to be influenced by a lysosome stabilizing compound such as cortisone or a labilizing factor such as streptolysin O. However, intact platelets were used, and the compounds may have been unable to penetrate the membranes.

In the present study, 3 lots of bacterial endotoxin had no demonstrable effect on the staining of human platelet granules. However, when heparinized rabbit platelets were used, aggregation occurred while granules remained brightly stained. This is in contrast to the degranulation of rabbit platelets by endotoxin which has been reported when examined by electron microscopy(8). Marcus and Zucker (9) reported that human and monkey platelets are not clumped by antigen-antibody plasma factor complexes and quoted unpublished results that endotoxin clumps rabbit platelets, but not human platelets. Des Prez(10) used 5-hydroxytryptamine release as an index of platelet damage by endotoxin, thrombin, and antigen-antibody complexes. He found that damage produced by thrombin was superficially similar to that produced by endotoxin, but that different mechanisms were involved for the 2 substances. Using rabbit platelets, indications were that antigen-antibody and endotoxin injury may be identical and secondary to platelet phagocytosis. The present investigations indicate that different mechanisms may be concerned in the reactions of human platelets with heterologous antisera, human antibody to platelets, and endotoxin and that injury with these is different from that mediated by thrombin.

In addition to exposure to thrombin and platelet antibody, the staining of platelet granules could be inhibited by incubation at low temperatures or an acid pH; the effects were reversible. The membrane may have been altered temporarily so that dye penetration was impossible; but whatever the mechanism involved, the 2 types of treatment were reversible even after as long as 10 days of storage with the standard ACD anti-coagulant.

The aggregating agents ADP and adrenaline as well at ATP, AMP, and adenosine had no effect on dye uptake. This finding is in agreement with that of Marcus and Zucker (11) who reported that degranulation occurs with thrombin and not with ADP.

It was hoped that these studies on dye uptake by platelets granules would provide an index of the effects of various conditions in the platelet environment on platelet structure and perhaps correlate such changes in structure with platelet function. However, normal staining of platelet granules *(in vitro)* after storage does not correlate with the failure of platelets stored for 10 days to survive *(in vivo)* when transfused.

*Summary.* The uptake of the fluorescent dye euchrysine by human platelets is not affected by the aggregating agents ADP and adrenaline or by endotoxin, but is lost after exposure to anti-platelet and anti-red cell

stroma serum. Granules of platelets fail to take up the dye at low temperatures and at an acid pH, both of these conditions being reversible. Platelets stored for 10 days at 5°C had normal staining of their granules.

1. Sokal, G., Thromb. Diath. Haemorr., 1963, v10, 235.
2. Balboa, R. S., Gajewski, M., Pawlowski, J. M., Greenwalt, T. J., Johnson, S. A., ibid., 1963, v10, 9.
3. Dausset, J., Vox. Sang., 1954, v4, 204.
4. Coombs, R. R. A., Bedford, D., ibid., 1955, v5, 111.
5. Marcus, A. J., Zucker-Franklin, D., Safier, L. B., Ullman, H. L., J. Clin. Invest., 1966, v45, 14.
6. De Duve, C., in Lysosomes, A. V. S. de Reuck & M. P. Cameron, eds., Little, Brown & Company, Boston, 1963, 1.
7. Allison, A. C., Mallucci, L., Lancet, 1964, v2, 1371.
8. Spielvogel, A. R., Blood, 1966, v28, 985.
9. Marcus, A. J., Zucker, M. B., The Physiology of Blood Platelets, Recent Biochemical, Morphologic and Clinical Research, Grune & Stratton, Inc., New York, London, 1965, 59-60.
10. Des Prez, R. M., Bryant, R. E., J. Exp. Med., 1966, v124, 971.
11. Marcus, A. J., Zucker, M. B., ibid., 35.

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## Developmental Changes in Dehydrogenase Activities in Rabbit Eggs.* (32586)

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Cytochemical techniques have been employed to study developmental changes occurring during maturation, fertilization and cleavage of several vertebrate eggs. The cytochemical characteristics of many of mammalian egg components, DNA, RNA, lipids and polysaccharide staining-positive materials, depend on the species and stage of development(2,10,11,12,14).

The metabolic pathways are markedly unique at different developmental stages (8,15). Studies in mammalian eggs have demonstrated the existence of multiple enzyme systems responsible for the respiratory activity in the rat(1,18,20) and in the rabbit (5,6,7,17). Some enzymes (phosphates and dehydrogenases) have been histochemically identified(3,4,13,21) and their activities appear to vary with the stage of cleavage; moreover their intracellular localization is different in the inner cell mass and trophoblast cells, perhaps reflecting the specific function of the cells.

To date, little is known about the dehydrogenase systems in the egg. Thus, this investigation presents a histochemical analysis of various dehydrogenase activities or their role in energy production during very early developmental stages of the rabbit embryo. From the data the possible role(s) of these enzymes in metabolism is discussed.

*Material and methods.* Adult New Zealand White rabbits weighing 7 to 10 lb were bred to fertile bucks, injected intravenously with 15 i.u. of HCG (Hafez, 1961) and sacrificed at specified times.

The different dehydrogenase activities were determined by a modification of Nachala's method (1957); the concentration of substrate was 0.1 M, the concentration of the electron acceptor was 0.2%, and the incubation period was 30 to 90 minutes. 5 to 7 eggs were used per culture vessel, which contained 0.3 to 0.5 ml of the incubation medium (1 ml of 0.1 M phosphate buffer at pH 7.4 and 1 ml of 0.2% nitroblue tetrazolium per

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