Avian thrombocyte aggregation and shape change in vitro

ROBERT A. GRANT AND MARJORIE B. ZUCKER
Department of Pathology, New York University School of Medicine, New York City 10016

METHODS

Blood was obtained from healthy, adult, white leghorn roosters by a scalp infusion set equipped with a 19-gauge needle (Jelco Labs, Raritan, N.J.). Venipuncture was performed on either the wing vein of restrained, unanesthetized birds or a surgically exposed jugular vein of anesthetized birds. Anesthesia was induced by intramuscular injection of 2.8 ml/kg of Army anesthesia (Frank E. Lentz Co., Philadelphia). The blood was collected at room temperature either into empty 12 x 75 mm polypropylene test tubes (Falcon Plastics, Oxnard, California) or into siliconized glass test tubes containing 0.11 vol of isotonic 2% neutralized EDTA. Each tube of anticoagulated blood was mixed by rapidly rolling it back and forth 15 times between the palms of the hands. The tubes were left at room temperature throughout the experiment. Several drops of the first and last blood to pass from the vein through the tubing were collected directly in polypropylene tubes containing citrate-Formalin fixative (28). These samples were later examined to determine the number and condition of thrombocytes at the beginning and end of the collection.

Clotting times were determined on native or recalcified, EDTA-treated whole blood in polypropylene tubes at room temperature. EDTA-treated blood, 0.1 ml, was added by siliconized pipette to each of a series of polypropylene tubes containing 10 µl of the reagents. All determinations were done in triplicate. The samples were usually shaken horizontally for 2 min at room temperature, then immediately mixed with at least 1 ml of Formalin fixative. Enough additional fixative was then added to dilute the blood 1:25, and the fixed samples were stored at room temperature. Values given in the text represent final concentrations. ADP disodium, AMP, and adenosinc (Sigma Chemical Co., St. Louis, Mo.) were all diluted in isotonic saline. Heparin (Panheprin, Abbott Laboratories, North Chicago, Ill.) was added to either native or EDTA-treated whole blood.

The number and condition of thrombocytes were ascertained as follows: The sedimented fixed whole blood samples were remixed; 0.1 ml of each was removed and added to 0.4 to 0.6 ml of fixative. The suspensions were placed in counting chambers. The number of thrombocytes and spherical cells (see below) were counted in 1 mm² on each side of the chamber with a phase-contrast microscope and the results expressed as the number per microliter of blood. We also noted the appearance of the cells and the presence of aggregat--
THROMBOCYTE AGGREGATION

The morphology of Formalin-fixed cells was unaltered by storage in the fixative at room temperature. Results are given as mean values ± standard deviations. Statistical significance was calculated by the t test on paired data.

RESULTS

The Formalin-fixed avian blood cells were identified by the same criteria used in Wright’s-stained smears (14). Erythrocytes were readily identified by their number, size, and shape. Typical thrombocytes were ovoid, slightly smaller than erythrocytes, and had an ovoid nucleus; the faintly textured cytoplasm often contained what appeared to be vacuoles and one or more granules. A typical thrombocyte in a Wright’s-stained smear is shown in Fig. 1A; thrombocytes in Formalin-fixed samples are shown in Fig. 1B and C. Many typical thrombocytes were seen in native blood allowed to flow directly into fixative, as well as in EDTA-treated blood kept at room temperature. In contrast, typical thrombocytes were not seen in EDTA-treated blood kept at 4°C for 10 min.

Large and small spherical cells without visible nuclei were also seen in Formalin-fixed samples. They could be easily differentiated from typical thrombocytes by their shape and apparent lack of nucleus. Since no anuclear cells were seen in Wright’s-stained smears, the nucleus was presumably present but obscured by cytoplasm. Large spherical cells, almost the size of erythrocytes and with a textured surface, were probably granular leukocytes (Fig. 1D); they were notably larger than thrombocytes. The small spherical cells had a dense margin and a translucent center; one is shown in Fig. 1E.

EDTA-treated blood shaken for 2 min at room temperature contained an average of 30,000 typical thrombocytes and 16,000 small spherical cells per microliter (Table 1). The latter probably represent lymphocytes. These values agree well with those reported (14). When 10 μM ADP was added and the blood shaken, the number of typical thrombocytes decreased, and correspondingly more small spherical cells were noted (Table 1). Similar treatment with 5 μM AMP had no such effect. ADP-induced conversion of typical thrombocytes into small spherical cells was inhibited by incubating the blood for 5 min with 500 μM adenosine prior to treatment with ADP (Table 2). The shape of typical thrombocytes was unaffected by placing EDTA-treated blood at 4°C for 10 min.

Occasionally, simple shaking of control, EDTA-treated blood for 2 min at room temperature converted typical thrombocytes into small spherical cells. This change was inhibited in blood incubated with 500 μM adenosine for 5 min prior to shaking (Table 3).

Shaking 0.1 ml of blood for 2 min at room temperature increased blood pH from 7.4 to 7.6. We therefore performed a series of experiments in which the blood samples were shaken in sealed test tubes filled with 5% CO2. ADP and occasionally simple shaking induced the usual change in typical thrombocyte shape even without an increase in blood pH.

Thrombocyte aggregation was not observed when EDTA-treated blood was shaken for 2 min with 10–50 μM ADP but occurred when more than 5 mM CaCl2 was added instead. Aggregates consisted of 5–50 cells, some of which were difficult to identify. Many were small and large spherical cells. Such a clump is seen in Fig. 1F. After CaCl2 addition, EDTA-treated blood contained a total of 16,000 ± 11,000

---

FIG. 1. Photomicrographs of avian blood showing: A: Wright’s-stained smear; B–F: Formalin-fixed samples under phase contrast. Cells not designated by arrows are erythrocytes. A: thrombocyte (1,500X); B: typical thrombocyte (640X); C: typical thrombocyte (400X); D: large spherical cell (400X); E: small spherical cell (400X); F: aggregate (400X).
treated avian whole blood thrombocytes by adenosine; SSC, small spherical cells.

TABLE 3. Thrombocyte shape change induced by shaking and its inhibition by adenosine

<table>
<thead>
<tr>
<th>Exp No.</th>
<th>Unshaken</th>
<th>Shaken</th>
<th>Shaken With Adenosine (500 µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of TT + SSC, 10³/µl</td>
<td>TT, %</td>
<td>No. of TT + SSC, 10³/µl</td>
</tr>
<tr>
<td>1</td>
<td>*</td>
<td>*</td>
<td>53</td>
</tr>
<tr>
<td>2</td>
<td>*</td>
<td>*</td>
<td>36</td>
</tr>
<tr>
<td>3</td>
<td>51</td>
<td>73</td>
<td>43</td>
</tr>
<tr>
<td>4</td>
<td>60</td>
<td>56</td>
<td>50</td>
</tr>
</tbody>
</table>

TT, typical thrombocytes; SSC, small spherical cells. * Not done.

free typical thrombocytes and small spherical cells per micro-liter compared to a total of 50,000 ± 11,000 typical thrombocytes and small spherical cells per micro-liter in samples shaken without CaCl₂ (n = 12; P < 0.01). Such recalcified blood samples clotted in 22 ± 10 min. Calcium-induced aggregation was not inhibited by pretreating the blood with 500 µM adenosine or even with 10 units of heparin per milliliter, which completely inhibited clotting. Aggregation also occurred in recalcified blood shaken under CO₂ with no change in blood pH. An attempt was made to ascertain whether ADP induced aggregation of avian thrombocytes in vitro. The results of adding ADP (10 µM) to EDTA-treated blood containing either saline or 5 mM CaCl₂ are shown in Table 4. There was no statistically significant difference in the total number of typical thrombocytes and small spherical cells between samples containing both ADP and CaCl₂ and those containing only CaCl₂. The inability of ADP to cause or potentiate aggregation was not due to its rapid disappearance since when 1 µM ADP was added to native plasma, 80% remained after 2 min at room temperature. It should be noted that while 5 mM CaCl₂ causes some aggregation, a more marked degree is caused by higher calcium concentrations.

TABLE 4. Effect of 10 µM ADP on EDTA-treated avian whole blood recalcified with 5 mM CaCl₂

<table>
<thead>
<tr>
<th>Exp No.</th>
<th>No. of TT + SSC, 10³/µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>70</td>
</tr>
<tr>
<td>2</td>
<td>39</td>
</tr>
<tr>
<td>3</td>
<td>38</td>
</tr>
<tr>
<td>4</td>
<td>41</td>
</tr>
<tr>
<td>5</td>
<td>23</td>
</tr>
</tbody>
</table>

TT, typical thrombocytes; SSC, small spherical cells. * Not done.

The thrombocytes also aggregated in freshly collected native blood shaken for 2 min at room temperature, but not in unshaken native blood. The aggregates were similar to those observed in shaken, recalcified, EDTA-treated blood. Aggregation occurred in blood shaken in paraffin-coated and siliconized test tubes, and was not inhibited by adenosine, heparin, or maintenance of physiological blood pH. Shaken native blood clotted in about 50 ± 13 min (n = 7); heparinized blood did not clot.

DISCUSSION

The mechanism by which shaking causes thrombocyte aggregation in recalcified or native avian whole blood is not understood. Thrombin does not appear to be responsible since aggregation occurred much more rapidly than coagulation and was not inhibited by heparin. The fact that thrombocytes circulate unclumped in vivo although blood contains ionized calcium suggests that the trauma of blood collection makes these cells react to shaking or that in vitro shaking does not resemble any activity naturally encountered by the cells while passing through uninjured blood vessels. No evidence of trauma to the cells was noted after careful blood collection; the majority of thrombocytes retained their typical form and there was no aggregation. Neither the surface of the tubes used in these experiments nor the change in pH that was noted appears to be important.

In some respects thrombocyte aggregation resembles the platelet aggregation caused by adding divalent cations such as calcium or magnesium to citrated or EDTA-treated platelet-rich plasma or washed platelets (5, 8, 9, 15, 19, 20). Silver (19) found that aggregates appeared about 2 min after recalcifying citrated platelet-rich plasma at room temperature, whereas clotting required about 14 min, suggesting that thrombin was not responsible. In other experiments, he found that heparin prevented clotting but not calcium-induced aggregation (20). However, calcium-induced platelet aggregation, unlike thrombocyte aggregation, is inhibited by adenosine (8, 15).

ADP appears to have a small role, if any, in thrombocyte aggregation for several reasons: 1) ADP cannot potentiate the aggregation noted when suboptimally recalcified, EDTA-treated blood is shaken (Table 4). 2) Adenosine, an inhibitor of ADP-induced human platelet aggregation (5, 21) fails to inhibit calcium-induced thrombocyte aggregation. 3) Thrombin-induced thrombocyte aggregation is not...
inhibited by phosphoenol-pyruvic acid and pyruvate kinase (3), a system which inhibits thombin-induced platelet aggregation by consuming ADP released from the platelets (11). If ADP neither aggregates nor mediates the aggregation of nonmammalian thrombocytes, a hemostatic mechanism quite unlike the one thought to occur in mammals must be postulated. Although ADP is unable to cause aggregation in vitro, it induces a profound change in morphology, converting typical thrombocytes to small spherical cells in EDTA-treated blood. Platelets also change their shape when exposed to ADP (29). Thrombocyte shape change induced by either ADP or shaking was inhibited in blood incubated with adenosine. These morphologic observations help to explain the numerous reports of different types of thrombocytes within one species. If stimuli such as ADP and shaking can induce shape changes, venipuncture, centrifugation and exposure to glass microscope slides, for example, may well induce similar or more drastic changes. Our observations and those of Carlson et al. (6) in chickens and of Srivastava (22) in teleosts suggest that thrombocytes are discoid in shape while free and circulating. Our ability to detect shape change and early aggregation was facilitated by our choice of EDTA-treated whole blood rather than TRP, and by the combined use of Formalin fixation and phase-contrast microscopy.

We gratefully acknowledge the assistance of Earl Lockert. This work was carried out in partial fulfillment of the requirements for the Master of Science degree awarded to R. A. Grant by Long Island University, New York City, and was partially supported by Public Health Service Grant HL-12859.

Received for publication 16 October 1972.

REFERENCES


