Spontaneous Platelet Aggregation in a Hereditary Giant Platelet Syndrome (MPS)

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The characteristics of spontaneous platelet aggregation (SPA) in a hereditary giant platelet syndrome (Montreal platelet syndrome, MPS) are examined. SPA was quantitated by microscopy from the decrease in single platelets in platelet-rich plasma (PRP). In contrast to normal donors, a significant proportion (20–50%) of platelets in MPS whole blood and PRP occurred in microaggregates typically containing 2–6 disk-shaped platelets. Stirring MPS-PRP at 1000 rpm for 10 minutes further increased the fraction of platelets in aggregates by 10–170%, the percentage increase not being correlated to the donor's platelet count (5000–220,000 μl⁻¹). Normal platelets resuspended in MPS platelet-poor plasma (PPP) did not undergo SPA, whereas MPS platelets resuspended in normal PPP or Ca²⁺-free, fibrinogen-free Tyrode's continued to show SPA. The increase in SPA could be inhibited by 10 μM prostaglandin (PG) E₁, 150 mM ASA or glutaraldehyde or formaldehyde fixation; however, it was not inhibited by 10 nM PGI₂, and was only partially inhibited by 1 μM 2-chloroadenosine and 1–10 units/ml aprotase. SPA in Acid-citrate-dextrose–PRP was much less than in PRP; however, SPA reoccurred on returning the platelets to platelet-free plasma or Tyrode's. Platelet aggregation (PA) could be increased over that due to SPA alone by the addition of adenosine diphasphate, adrenaline, collagen, ionophore A-23187, arachidonic acid and ristocetin, with results suggesting that the response to these agents is normal. The ristocetin-induced increase in PA was completely blocked by an IgG specific for Bernard–Soulier syndrome. In contrast, MPS platelets had a reduced sensitivity to thrombin, which appeared to be more pronounced at low platelet counts. There was no correlation between the thrombin insensitivity and the extent of SPA. Total adenosine triphosphate (ATP) and thrombin-induced release of ATP and platelet factor 4 appeared normal for MPS platelets. The ultrastructural features of MPS platelets were within normal limits except for an increased frequency of granules. SPA was observed for 5/5 MPS donors, but only one of three MPS donors' platelets evaluated for glycoprotein I and sialic acid content showed any measurable reduction as compared with normal controls. The above observations point to the existence of an as yet undetermined anomaly of MPS plasma membrane related to a fibrinogen and Ca²⁺ independent form of platelet aggregation. (Am J Pathol 1984, 114:336–345)

SPONTANEOUS PLATELET AGGREGATION (SPA) has been described in a number of acquired platelet disorders and has been attributed to a leakage of adenosine diphosphate (ADP). In contrast, the occurrence of SPA in hereditary platelet disorders has received little attention. In 1963, Lacombe and d'Angelo described a thrombocytopenic, macrothrombopathia characterized by SPA, dominant inheritance, the appearance of giant platelets on peripheral blood smears, a prolonged bleeding time, normal clot retraction, and thromboplastin formation. This syndrome was subsequently renamed Montreal platelet syndrome (MPS) and further characterized by normal amounts of platelet plasma membrane, but a hypervolumetric shape change associated with platelet activation. Here we focus on the nature of SPA in MPS.

The results demonstrate that MPS SPA is distinct from that observed in acquired disorders and is associated with an intrinsic platelet membrane defect. This membrane defect is not correlated with the

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amount of membrane glycoprotein Ib nor sialic acid. Future characterization of MPS platelet membranes may provide insights into the mechanism(s) whereby “sticky” sites are generated by aggregating agents in normal platelets.

Materials and Methods

Case Histories

Normal donors were chosen from healthy men and women between the ages of 18 and 25. This study focuses on 3 affected members of an MPS family: 2 sisters (I.T., 34 years; L.T., 37 years) and their brother (P.T., 29 years). Clinical descriptions of these donors have been published previously, and there have been no clinically significant episodes of hemorrhage or bruising tendency since that time. Preliminary studies on 2 additional affected family members—the mother (65 years) and her granddaughter (daughter of L.T., 13 years)—have yielded results similar to those reported here (data not shown). Details of MPS platelet size on blood smear and in platelet-rich plasma (PRP) have been discussed previously and are summarized in Table 1.

Platelet Preparations

Blood (9 vol) was drawn by venipuncture into 3.8% sodium citrate solution (1 vol), pH 7.4, 37 C, and PRP (cPRP) prepared as described previously. Aster citrated blood was prepared by adding 9 vol of blood to 1 vol of Aster solution composed of 4.48% sodium citrate, 2.73% citric acid, and 2% glucose and processed as above. Two methods were used to concentrate platelets from Aster PRP: 1) isolation on a metrizamide gradient and 2) pelleting by centrifugation at 1900 g for 15 minutes. Platelets prepared in this manner and resuspended into Ca²⁺-free Tyrode’s, pH 7.4, did not aggregate in response to 10–100 μM ADP even with the addition of 1 mM CaCl₂. Platelet-poor plasma (PPP) and platelet-free plasma (FPF) were prepared from cPRP as described previously. Resuspended platelets were allowed to stand for 30 minutes at 37 C and under 5–6% CO₂ before the addition of aggregating agents.

Platelet Aggregation

Aggregation from transmittance changes was determined as previously described with the use of a Payton aggregometer. For aggregation evaluated by single particle counting, the aggregometer was used as a stirrer with cuvettes (6.9 x 45 mm) containing stir bars (6 mm x 1 mm) spun at 1000 rpm at 37 C and containing 0.1 ml platelet suspension. Solutions containing aggregating agent (1–5 μl) were quickly added with the use of a Hamilton syringe. Platelet aggregation was terminated by adding 4 vol 1% glutaraldehyde (vol/vol); this fixative does not influence the extent of platelet aggregation. Sampling times for platelet aggregation correspond to that for maximal increase in light transmission seen when the same agent was added to normal PRP. Platelet aggregation (%PA) was determined by counting the number of nonaggregated platelets with the use of a hemocytometer and the relation

\[
\%PA = \left(1 - \frac{N_t}{N_T}\right) \times 100% \tag{1}
\]

where \(N_T\), \(N_t\) are, respectively, the total number of platelets and number of free platelets at time t. In the absence of stirring, the MPS platelet aggregates tend to be small (see Figure 1), and typically there is no difficulty in determining the numbers of platelets in the aggregates and hence \(N_T\).

MPS platelet aggregating responses were compared to those seen in normal cPRP diluted with PPP or FPF to give the same total platelet count.

Platelet Release From α-Granules and Dense Granules

Dynamic release of adenosine triphosphate (ATP) from platelets was measured with a Whole Blood Lumiaggregometer (Chronolog Corp., Havertown, Pa). Luciferin-luciferase reagent (Chronolume, Chronolog) was added to 1 ml of platelet suspension 3 minutes before addition of aggregating agent. Measurable release was obtained only for washed concentrated platelets (pelleted from Aster citrated PRP) since the platelet count was too low in MPS whole blood or PRP (\(N_T \approx 40,000 \mu l^{-1}\)). The measured response was standardized by the addition of freshly prepared ATP (final concentration, 1 μM).

Samples for measuring releasable ATP and platelet factor 4 (PF₄) were prepared from platelets pelleted by centrifugation from Aster citrated PRP and resuspended into Ca²⁺-free Tyrode’s, pH 7.4. Platelet suspensions from normal and MPS donors were adjusted to the same platelet count (\(\approx 200,000 \mu l^{-1}\)). Total releasable ATP and PF₄ was measured from samples treated with 5 units/ml thrombin for 3 minutes. The determinations were done in triplicate, the samples pooled (total 1.2 ml), placed in ice-water for 30 minutes, then spun at 15 C for 30 minutes at 2500 g. The top 0.3 ml of the supernatant was frozen for PF₄ assays, and the next 0.6 ml was frozen for ATP assays. ATP was measured with the use of lucif-
erin-luciferase (Sigma Chemical Co.), standard ATP dilutions, and a scintillation counter as a luminescence detector. PF3 was measured with the use of a radioimmunoassay (Abbott Laboratories, North Chicago, Ill) and was performed by Dr. Gilles Latourelle (Montreal Heart Institute) on freshly thawed samples that had been diluted 10-50-fold with Ca²⁺-free Tyrode's, pH 7.4.

Electron Microscopy

Blood for platelet ultrastructural studies was mixed immediately with citrate-citric acid dextrose, pH 6.5 (9.3 mM sodium citrate, 7.0 mM citric acid and 140 mM dextrose) in a ratio of 9 parts blood to 1 part anticoagulant. cPRP was separated from whole blood by centrifugation at room temperature for 20 minutes at 100 g. Samples for electron microscopy were combined with an equal volume of 0.1% glutaraldehyde in White's saline, pH 7.3 (a 10% solution of a 1:1 mixture of 1) 2.4 M NaCl, 0.1 M KCl, 46 mM MgSO₄, 64 mM Ca(NO₃)₂·4H₂O, and 2) 0.13 M NaHCO₃, 8.4 mM NaHPO₄·7H₂O, 3.8 mM anhydrous KH₂PO₄, and 0.1 g/l phenol red). After 15 minutes at 37 C the samples were sedimented to pellets, and the supernatant was discarded and replaced with 3% glutaraldehyde in the same buffer. Fixation was continued at 4 C for 60 minutes. The cells were then washed in buffer and combined with 1% osmic acid in veronal acetate (0.02 N HCl a 20% solution of a stock buffer solution containing 0.14 M sodium barbital, 0.145 M sodium acetate 3H₂O, and 6.8% solution of a stock salt solution containing 1.7 M NaCl, 54 mM KCl, and 18 mM CaCl₂). After exposure to the second fixation for 1 hour the cells were dehydrated in a graded series of alcohol and embedded in Epon 812. Contrast of thin sections cut from plastic blocks on an ultramicrotome was enhanced with uranyl acetate and lead citrate. Observations were made in a Philips 301 electron microscope.

Chemicals

Thrombin (purified from human plasma, specific activity: 4000-5000 NIH units/mg), 2-chloroadeno-

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Table 1

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>I.T.</th>
<th>L.T.</th>
<th>P.T.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet count (μl⁻¹)</td>
<td>250,000-700,000</td>
<td>5,000-15,000</td>
<td>27,000-120,000</td>
<td>20,000-41,000</td>
</tr>
<tr>
<td>Mean diameter* on blood smear (μm)</td>
<td>1.8 ± 0.21</td>
<td>3.2</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Vr (μl)†</td>
<td>4.0-7.76</td>
<td>8.6</td>
<td>7.1</td>
<td>7.3</td>
</tr>
<tr>
<td>Vo (μl)†</td>
<td>4.0-7.88</td>
<td>6.0</td>
<td>4.2</td>
<td>4.4</td>
</tr>
<tr>
<td>%PAA†</td>
<td>No stirring</td>
<td>16 ± 11</td>
<td>32 ± 1</td>
<td>36 ± 3</td>
</tr>
<tr>
<td></td>
<td>42s stirring</td>
<td>41 ± 20</td>
<td>55 ± 13</td>
<td>42 ± 11</td>
</tr>
<tr>
<td></td>
<td>10s stirring</td>
<td>64 ± 2</td>
<td>55 ± 10</td>
<td>58 ± 13</td>
</tr>
</tbody>
</table>

* Data on platelet size for MPS donors has been partially presented previously. Platelet mean volume (Vr) and mean discocyte volume (Vo) are identical in freshly fixed blood and citrated PRP, 37 C, but substantially larger in Aster citrate.
† The disproportion between Vr and Vo (Vr/Vo = 1.12 ± 0.05 for 27 normal donors, mean ± 1 SD) implies that MPS discoid platelets are abnormally large. This observation has been termed "hypervolumetric" shape change and is also observed for platelets from Bernard-Soulier syndrome but not for platelets from a number of other hereditary giant platelet syndromes, including the May-Hegglin anomaly. Platelet shape change is independently controlled from the volume change and can occur without any accompanying volume change. Platelet shape change is not an artifact of the technique.
‡ Data for 4 normal donors and is represented as the mean ± 2 SD.
§ Data for 35 normal donors and has been represented as the mean (minimum–maximum).
∥ %PA calculated from equation 1. Data for MPS donors are represented as the mean ± 2 SD and for each donor represent a minimum of 4 measurements made on 4 separate occasions.
PLATELET AGGREGATION

Table 2—Relative Role of Plasma and Platelets in
MPS Spontaneous Platelet Aggregation

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Platelet count (μl⁻¹)</th>
<th>No stirring</th>
<th>42 seconds, stirring</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal washed platelets in MPS PFP</td>
<td>300,000</td>
<td>3%</td>
<td>3%</td>
</tr>
<tr>
<td>Normal PRP diluted 1:10 with MPS PFP</td>
<td>30,000</td>
<td>2%</td>
<td>2%</td>
</tr>
<tr>
<td>MPS-washed platelets in Normal FFP</td>
<td>25,000</td>
<td>38%</td>
<td>56%</td>
</tr>
<tr>
<td>Ca²⁺-free Tyrode's</td>
<td>230,000</td>
<td>43%</td>
<td>59%</td>
</tr>
</tbody>
</table>

Role of Plasma in SPA

Neither resuspending washed, concentrated platelets from normal donors into MPS PFP nor addition of MPS PPP to normal cPRP resulted in SPA (Table 2). On the other hand, MPS washed, concentrated platelets resuspended into either normal PFP or Ca²⁺-free Tyrode's continued to demonstrate SPA and to the same extent as observed in cPRP. These results indicate that SPA is an intrinsic MPS platelet property. Moreover, they imply that MPS SPA occurs independently of external fibrinogen and Ca²⁺.

Effect of Inhibitors on SPA

The stirring-dependent increase in MPS SPA could be completely blocked by fixation by glutaraldehyde or formaldehyde. Addition of 1 μM 2-chloroadenosine inhibited SPA by 41% for donors P.T. and I.T. (for calculation of percentage of inhibition of SPA see legend to Figure 2). Although 1 unit/ml apyrase caused a 30–50% inhibition of SPA, increasing the apyrase concentration 10-fold did not further inhibit SPA and indeed was generally less effective (30% inhibition of SPA with 1 unit/ml apyrase and 20% inhibition with 10 units/ml for donor P.T.). This observation suggests that the partial inhibition of SPA caused by 2-chloroadenosine and apyrase is not simply an inhibition of ADP–platelet interactions.

Results

Spontaneous Platelet Aggregation

In contrast to cPRP prepared from normal donors, a significant proportion of platelets in MPS cPRP occur in microaggregates, which typically contained 2–6 disk-shaped platelets, though on occasion, much larger aggregates could be observed (see Figure 1 and Table 1). Stirring MPS cPRP at 1000 rpm for 10 minutes increased the fraction of platelets in the microaggregates. The proportion of platelets aggregated in cPRP as well as the increase in SPA upon stirring was independent of the different MPS donor's platelet count (range 5000–73,000 μl⁻¹). Incubating MPS cPRP at 37 C led to a slow increase in %PA upon standing (10–20% further increase in 2–3 hours).

The same proportion of platelets were aggregated in whole blood whether it was immediately fixed upon collection with buffered glutaraldehyde (30% PA) or when citrate or EDTA were utilized as anticoagulants prior to fixation (32% and 26% PA, respectively). In addition, no significant differences were observed between MPS cPRP and MPS cPRP plus hirudin and 2 mM CaCl₂. These observations indicate that MPS SPA is not an artifact introduced by preparation.

Figure 2 — Inhibition of the stirring-dependent increase in MPS platelet aggregation by ASA and PGE, and PGI₂. Data is for donor P.T., and each point is the mean of two measurements. The percentage of inhibition was determined from the relation:

\[
\% \text{ inhibition} = \left( \frac{\% \text{ PA}_b - \% \text{ PA}_0}{\% \text{ PA}_b} \right) \times 100\%
\]

where (%PA)₀, (%PA)₀, and (%PA)₀ are, respectively, the percentage of platelet aggregation determined from equation 1 without stirring and after 10 minutes' stirring in the presence and absence of inhibitor. Donor I.T. (data not shown in Figure) gave 69% inhibition of SPA by 1 μM PGE, and 28% inhibition by 1 unit/ml apyrase on one occasion.
Table 3—Influence of Aster Citrate on MPS
Spontaneous Platelet Aggregation

<table>
<thead>
<tr>
<th>Spontaneous platelet aggregation (no stirring)</th>
<th>Donor P.T.</th>
<th>Donor L.T.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrated PRP (pH 7.4)</td>
<td>38%</td>
<td>28%</td>
</tr>
<tr>
<td>Aster PRP (pH 6.8)</td>
<td>8%</td>
<td>6%</td>
</tr>
<tr>
<td>Aster PRP platelets re-suspended in Ca²⁺-free Tyrode’s (pH 7.4)</td>
<td>43%</td>
<td>—</td>
</tr>
<tr>
<td>MPS PRP (pH 7.4)</td>
<td>—</td>
<td>38%</td>
</tr>
</tbody>
</table>

* Resuspended to a platelet count of 230,000 µl⁻¹.
† Resuspended to same platelet count as observed in citrated PRP (ie, 20,000 µl⁻¹).

This is in contrast to SPA in acquired platelet disorders, which appears to be a reflection of ADP release.

The effects of ASA, PGE₁, and PG₁₂ on inhibition of SPA are examined in Figure 2. ASA (150 µM) and PGE₁ (10 µM) effectively block SPA. However, PG₁₂ did not inhibit SPA and at low concentrations appeared to enhance SPA. It is well recognized that PG₁₂ is generally a much more effective inhibitor of platelet aggregation than PGE₁. In view of this observation, the observed inhibition of SPA by ASA and PGE₁ presumably arises via nonspecific interactions.

All of the above inhibitors failed to dissociate microaggregates which had formed prior to their addition.

Table 4

<table>
<thead>
<tr>
<th>Platelet aggregation (%)</th>
</tr>
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<tbody>
<tr>
<td>Agent</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Thrombin‡</td>
</tr>
<tr>
<td>ADP</td>
</tr>
<tr>
<td>Ristocetin</td>
</tr>
<tr>
<td>Collagen</td>
</tr>
<tr>
<td>A-23187</td>
</tr>
<tr>
<td>Adrenaline</td>
</tr>
</tbody>
</table>

* Corrected %PA, (%PA)corr, determined by the relation:

\[
\frac{(%PA)corr}{100} = \frac{(\% PA)A - (\% PA)R}{(\% PA)R}
\]

where (%PA)A, (%PA)R are, respectively, %PA at time t in the absence and presence of aggregating agent.

† Normal PRP diluted with normal PPP to give same platelet count as seen for MPS PRP.
‡ Sampling times correspond to time for maximum increase in light transmission following addition of agent to normal PRP.
§ Absence of response to thrombin was observed in 4/4 occasions for L.T.; 4/5 occasions for I.T., and 0/5 occasions for P.T. Increasing thrombin concentration to 0.5 units ml⁻¹ did not increase %PA.
‖ Mean ± 1 SD.
§ Mean ± 1 SD.

Effect of Aster Citrate on SPA

Isolation of MPS platelets in Aster citrate (final pH 6.8) almost eliminated SPA (Table 3). However, on pelleting platelets from Aster citrate and resuspending them into either MPS PPP or Ca²⁺-free Tyrode’s at pH 7.4, SPA returned to levels seen in cPRP. Aster citrate was found to inhibit ADP-induced platelet aggregation for normal PRP prepared from Aster citrated blood or upon its addition to washed platelets in Ca²⁺-albumin–Tyrode’s.

Effect of Aggregating Agents on SPA

Addition of ADP, adrenalin, collagen, ionophore A-23187, and ristocetin to MPS PRP increased MPS platelet aggregation (Table 4). The increase in platelet aggregation induced by these agents when corrected for SPA was similar to that observed for normal platelets at the same platelet count. The ristocetin-induced increase in platelet aggregation could be blocked by addition of IgG (BSS). These results suggest that the response of MPS platelets to these aggregating agents is within normal limits.

In contrast, 0.2 units/ml thrombin added to MPS-PRP either completely failed to induce an increase in platelet aggregation or else induced a smaller increase than observed in studies of normal platelets at a similar platelet count. Increasing the thrombin concentration to 2 units/ml increased the platelet aggregation to ~ 80% at 42 seconds for donor PT. When MPS
platelets were pelleted from Aster citrate and resuspended into Ca²⁺-free Tyrode's, pH 7.4, at a platelet count of 200,000 µl⁻¹, a "normal" light transmission response was obtained with 0.2 units/ml thrombin (Figure 3). This is consistent with the observations for normal platelets that there is an approximately 10-fold difference in thrombin sensitivity between cPRP and washed platelets (W. Hong and M. Frojmovic, unpublished observations). However, rediluting these concentrated platelets with Tyrode's to give the original platelet count gave the reduced thrombin response as seen in cPRP. These results suggest that MPS platelets have a reduced sensitivity to thrombin, which appears to be more marked at low platelet counts.

Addition of 1 vol normal PFP to MPS PRP or resuspension of MPS platelets into normal PFP did not alter the response of MPS platelets to 0.2 units/ml thrombin (%PA not increased over that due to SPA alone in all cases). Normal platelets resuspended into MPS PFP showed a normal sensitivity to thrombin (%PA of 35 and 34 at 42 seconds after addition of 0.2 unit/ml thrombin, respectively, for resuspension into normal and MPS PFP; platelet count, ~40000 µl⁻¹).

Figure 3 — Aggregation of washed MPS platelets by A) arachidonic acid (0.45 mM AA) and B) thrombin (0.2 units Th ml⁻¹). Light transmission in the aggregation tracings increases from 28% to 70%, with <1% increase seen with stirring in the absence of any activator. Platelets were pelleted from Aster citrate and resuspended in Ca²⁺, fibrinogen-free Tyrode's, to a platelet count of 200,000 µl⁻¹. The results were independent of readdition of 1 mM CaCl₂.

Figure 4 — MPS platelets fixed in glutaraldehyde and osmic acid without ferricyanide favors preservation of dense bodies (i), which are normal in number in MPS. (x 27,500) A — MPS platelets containing giant granules (GG). (x 27,500)
Table 5—Comparison of Thrombin-Induced (5 units/ml) Release of ATP and Platelet Factor 4 (PF₄) for Normal and MPS Donors

<table>
<thead>
<tr>
<th>Thrombin-induced release</th>
<th>Normal (n = 1)</th>
<th>MPS (P.T.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP (µmol/10¹¹ platelets)</td>
<td>2.4*</td>
<td>2.5</td>
</tr>
<tr>
<td>PF₄ (µg/10⁹ platelets)</td>
<td>14.3</td>
<td>12</td>
</tr>
</tbody>
</table>

* Normal range of 1.9 ± 0.8 µmol/10¹¹ platelets reported by Meyers et al.²²

Thrombin-induced clot retraction appeared to be normal for MPS. The abnormal thrombin response seen for MPS cPRP was not correlated with the degree of SPA (correlation coefficient not significantly different from zero), suggesting that these two phenomena are not related to a common underlying platelet defect.

Dense Granules and Ultrastructures

Figure 4A demonstrates that MPS platelets contain dense granules whose number were found to be similar to that seen for normal platelets, suggesting that SPA is not related to any extensive release of dense granule contents prior to aggregating. The overall platelet ultrastructure, including microtubules and the dense tubular system, appeared to be normal, except for an increased frequency over normal platelets of giant granules (see Figure 4B).

Thrombin and Arachidonic-Acid-Induced Release From α-Granules and Dense Granules

The extent of release of ATP and PF₄ of MPS platelets induced by thrombin is within normal limits (see Table 5). Moreover, Figure 5 shows that the time course of MPS platelet ATP release induced by 0.2 units/ml thrombin is comparable to that obtained for normal donors (not shown).¹⁸ These observations suggest that there is no suppression in the thrombin-induced MPS platelet release reaction. Indeed, freeze–thaw (IX)-induced release of ATP gave identical results as mean values for total platelet ATP reported (5.7 versus 5.5 ± 1.23 µ moles/10¹¹ platelets).

Arachidonic acid (AA; 0.45 mM) gave 50% and 16% of the ATP and PF₄ release, respectively, observed with thrombin for MPS, shown in Table 5, though yielding a full aggregation response (see Figure 3). These values were, however, well above control values obtained with normal washed platelets (0.3 µmol ATP/10¹¹ platelets and 0 PF₄ µg/10⁹ platelets), demonstrating that AA-induced dense granule release for MPS platelets is even more efficient than for normal platelets.

Freeze-Fracture Studies of MPS Platelet Membranes

The P face of MPS platelet membrane is shown in Figure 6A, and the E face of another, in Figure 6B.

![Figure 5 — Release and aggregation of washed, concentrated MPS platelets induced by thrombin. A — ATP secretion measured by luminescence, with 1 µM ATP added at t. B — Aggregation measured with the aggregation; in both cases, CaCl₂ and thrombin were added as shown at t. Omission of CaCl₂ did not significantly alter the ATP release kinetics.](image-url)
Intramembranous particles of various sizes are apparent on both faces. Approximately 500 particles per square micron are evident on the E face and half that number on the P face. These values are consistent with those obtained from evaluation of freeze-fractured normal platelet membranes.

**Discussion**

Our results demonstrate that SPA in MPS is an intrinsic platelet defect. The fact that 2-chloroadenosine, apyrase, ASA, and PGE, but not PGH, all inhibit MPS SPA to varying extents suggests that the mechanism of inhibition by these agents does not involve specific processes mediated by ADP, cyclooxygenase, and/or prostaglandin.

SPA occurs in Ca²⁺ and fibrinogen-free platelet suspensions, suggesting that the mechanism for SPA is distinct from that of normal platelet aggregation. Moreover, regardless of the platelet count, MPS SPA only recruits ~65% of the platelets and does not produce the light transmission responses seen in association with normal platelet aggregation. These observations suggest that only a certain proportion of the MPS platelet population is capable of SPA and that the aggregates so formed are small, ie, too small to significantly affect light transmission through stirred PRP. Although MPS SPA appears independent of any measurable spontaneous release from dense granules (lumi-aggregometer) or extrinsically added fibrinogen, it does require membrane fluidity as proposed for normal fibrinogen-dependent aggregation, based on the block of aggregation following fixation of platelets with glutaraldehyde or formaldehyde. The above observations, together with the abnormal nature of the membrane rearrangements that occur in MPS platelets during activation, suggest the possibility of an intrinsic membrane defect in MPS platelets. Two additional observations support this suggestion: 1) one MPS donor (the mother) developed an IgG anti-platelet antibody after receiving platelet transfusions, and 2) the face-on view of MPS disk-shaped platelets is abnormally light when viewed under phase contrast microscopy.

Preliminary measurements of membrane sialic acid and glycoproteins (GPs) have been made on MPS and control platelets concentrated on a metrizamide gradient, frozen in liquid nitrogen, freeze-dried and analyzed by Dr. A. T. Norden in Paris according to previously published methods. Donor P.T. had
platelets with normal amounts of sialic acid (6.2 mg/mg protein) and GP profiles indistinguishable from normal controls; donor LT's platelets did not reveal any GP abnormality, either; in contrast, donor LT had platelets with reduced sialic acid (5.1 ± 0.1 [n = 2 and 8 months apart] versus 6.8 ± 0.7 [n = 3 normal donors] mg/mg protein), with a specific reduction detectable only in the profile position corresponding to GP Ib (2 occasions) but no other significant differences seen in GP or polypeptide profiles. Donor PT's platelets also showed normal binding of 125I-Factor VIII von Willebrand factor in the presence of ristocetin, as well as normal binding of the monoclonal antibody (AN51) to GP Ib (G. Tobelem, personal communication), further suggesting normal GP Ib for this donor. The observed variability in GP and sialic acid did not correlate with the extent of SPA, suggesting that neither of these membrane components is directly related to the genesis of SPA.

The clinical syndromes reported in association with circulating platelet aggregates in acquired platelet disorders, eg, transient ischemic attacks, cardiac sudden death, have not been observed for MPS. It is possible that the size of MPS circulating platelet aggregates (on average, 4–6 platelets per aggregate) is too small to cause these complications. The contribution of SPA to MPS thrombocytopenia, ie, by aggregate sequestration in the microcirculation, is unknown.

In contrast to all other aggregating agents examined, MPS platelets had a reduced sensitivity to thrombin. An impaired thrombin response has also been observed for the Bernard–Soulier syndrome and for certain leukemic patients (P. Ganguly, personal communication). The impaired thrombin response was more pronounced at low platelet counts. Some investigators have postulated that thrombin-induced aggregation is dependent on the release of ADP. Thrombin-induced release of ATP and PF₄ from MPS platelets appeared normal (Table 5 and Figure 5). It is possible that the more impaired thrombin response of MPS platelets observed at low platelet numbers reflects decreased ADP binding, possibly secondary to reduced affinity and/or numbers of ADP binding sites. In addition, it has recently been shown that thrombin binding to platelets can occur independently of membrane GP Ib, suggesting a role for other as yet unidentified membrane (glyco) proteins.

A number of technical points concerning the evaluation of platelet function in platelet disorders are illustrated by our studies with MPS. First, the occurrence of SPA may be overlooked in the use of standard turbidometric measurements, which are known to be relatively insensitive to the presence of small platelet aggregates. Second, it may be necessary for one to study platelet responses to aggregating agents as a function of platelet count with particular emphasis on the range of platelet counts normally observed in thrombocytopenic patients in order to be able to reliably detect platelet dysfunction. Certainly for MPS donors observations of platelet function at their normal low platelet count may be more relevant in vivo. Third, it may be useful to examine thrombin responses for patients with possible platelet dysfunction. Finally, our observations demonstrate that heterogeneity can exist at the molecular level within the same clinical syndrome and between members of the same family, eg, variable sialic acid and GP Ib results. Heterogeneity in another hereditary giant platelet syndrome, Bernard–Soulier syndrome, has long been suspected.

References

13. Tang SS, Frojmovic MM: Inhibition of platelet function by antithrombotic agents which selectively inhibit

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