Platelet Sialic Acid and Platelet Survival After Aggregation by ADP

By M. A. Packham, M. A. Guccione, R. L. Kinlough-Rathbone, and J. F. Mustard

Some investigators have reported recently that platelet surface sialic acid is decreased during ADP-induced aggregation, whereas others have reported an increase. Since removal of sialic acid from the platelet surface shortens platelet survival, we have determined the survival of platelets that have been aggregated by ADP. We have also measured the amount of sialic acid in the suspending fluid of platelets after ADP-induced aggregation. ADP-induced aggregation did not cause the loss of sialic acid from rabbit platelets (which do not undergo a release reaction in response to ADP) nor from washed human platelets in a medium containing physiologic concentrations of calcium in which granule contents are not released. In a medium without added calcium, ADP caused the release of \(^{14}C\) serotonin (42.5% \(\pm\) 3%) from human platelets, but less than 4% of the sialic-acid-containing material was released. It seems likely that little of the releasable sialic acid of platelets is in the dense granules or the \(\alpha\)-granules. Thrombin (5 U/ml) released 90.0% \(\pm\) 3.4% of the serotonin from human platelets but only 20.6% \(\pm\) 7.4% of the total sialic-acid-containing material. Neuraminidase removed 42.3% of the total sialic acid, presumably from the platelet surface. Rabbit platelets that had been aggregated by ADP and deaggregated survived normally when returned to the circulation. This observation also provides evidence that they had not lost membrane sialic acid during aggregation and deaggregation.

**ADENOSINE DIPHOSPHATE (ADP)** is one of the principal agents that cause platelet aggregation, but the changes induced in platelets by ADP are not fully understood. Recently, several groups of investigators have examined possible alterations in human platelet surface sialic acid during ADP-induced platelet shape change and aggregation. Motamed et al.\(^1\) reported an increase in sialic acids removable by neuraminidase during the shape change of platelets. Peerschke and Zucker\(^7\) were unable to demonstrate any change in the amount of sialic acid available for cleavage by neuraminidase on human platelets when the platelets had been changed from their disc shape to spiny spheres by treatment with 5 \(\mu M\) ADP. However, Bunting et al.\(^1\) reported that platelets that had aggregated in response to ADP and then deaggregated took up less tritium than control platelets during labeling by periodate oxidation followed by borotritide reduction. They also reported a decrease in the total protein and sialic acid and a decrease in the amount of sialic acid per mg of protein upon ADP-induced aggregation; these results were obtained in the presence of aspirin, which should have blocked the release of granule contents. They attributed their findings to a generalized loss of sialic acid from membrane glycoproteins. In contrast, Wu and Ku\(^4\) reported an increase in surface sialic acid on human platelets that had been stimulated by ADP, collagen, or thrombin. However, they did not determine that release of sialic-acid-containing material from the platelet granules had not occurred in their experiments. We have shown previously that treatment of rabbit platelets with thrombin causes the release of sialic-acid-containing material from the platelets, presumably from the storage granules;\(^5\) other investigators have shown that thrombin releases material containing sialic acid from human platelets.\(^2,6,8\) In the present studies, the possibility of loss of sialic acid from human platelets under conditions in which ADP does not or does cause the release of granule contents was examined. In addition, the effect of ADP on rabbit platelet sialic acid was studied, since platelets from this species do not release granule contents upon aggregation with ADP.

In earlier studies we have shown that rabbit platelets from which surface sialic acid has been removed by treatment with neuraminidase in vitro do not survive upon return to the circulation of rabbits.\(^5,9\) In addition, platelets that have been treated with proteolytic enzymes (plasmin, chymotrypsin, trypsin) that remove periodic acid Schiff staining material from membrane glycoproteins show a shortened platelet survival.\(^9\) If platelet surface sialic acid or glycoproteins were removed by treating platelets with ADP, one would expect changes in the survival of platelets that had been exposed to ADP. Therefore, we have determined the survival of rabbit platelets aggregated by ADP in vitro, deaggregated, and returned to the circulation.

**MATERIALS AND METHODS**

Materials were obtained from the following suppliers: ADP from Sigma Chemical Co., St. Louis, Mo.; bovine thrombin (topical) from Parke, Davis & Co., Detroit, Mich.; human fibrinogen from Kabi, Stockholm, Sweden (it was treated with diisopropylfluoro-
phosphatase before use as described previously[10]; N-acetylneuramini-c acid for the preparation of sialic acid standard curves from General Biochemicals, Chagrin Falls, Ohio; neuraminidase (Vibrio cholerae strain Z-4 from GIBCO, Grand Island, N.Y.; enzyme activity 500 U/ml when 1 U is defined as the amount of enzyme required to release 1 μg of N-acetylneuraminic acid from human α1-acid glycoprotein in 15 min at 37°C); 14C-serotonin (57 mCi/m mole, 5-hydroxytryptamine-3-14C-creatinine sulfate) from Amersham/Searle, Arlington Heights, Ill.; and radioactive chromium (10Cr) as Na210CrO4, 100-400 mCi/mg Cr, from New England Nuclear, Boston, Mass.

Suspended levels in Tyrode solution containing apyrase and 0.35% albumin were prepared from rabbit or human blood as described elsewhere.[11,12] Apyrase was prepared by a slight modification[13] of the method of Molnar and Lorand[8] and was used at a concentration (1 μl/ml) that converted 90% of 0.25 μM ATP to AMP and adenosine in 13 min at 37°C. Human platelets were prepared in media with and without calcium; fibrinogen was added before ADP in the aggregation studies of human platelets.[10,12] Measurements of platelet aggregation by a turbidimetric technique and the release of platelet granule contents from platelets prelabeled with 14C-serotonin have been described by Greenberg et al.[5] The amount of sialic acid in the suspending fluid of untreated platelets was subtracted before the percent release or loss of sialic acid was calculated.

For the platelet survival studies, rabbit platelets were labeled with 10Cr as described by Greenberg et al.[5] washed, and resuspended in Tyrode solution containing apyrase and 0.35% albumin.[11,12] These platelets were aggregated by the addition of ADP (250 μM final concentration) with stirring at 37ºC. After 15 min, the platelets were removed by centrifugation, resuspended in fresh medium with apyrase, and incubated for 1 hr at 37ºC. During this time, deaggregation occurred (confirmed by microscopic examination). The platelets were removed by centrifugation and resuspended in fresh medium at a platelet count of 1.5 x 10⁹/cu mm. Control platelets were subjected to the same procedures except that Tyrode solution was added instead of ADP. Blood samples for the determination of platelet survival were taken as described previously. Platelet survival was calculated using Murphy’s gamma function.[15,16]

Total platelet sialic acid and the amount of sialic acid in the supernatant fluid of platelets that had been exposed to ADP was measured by the method of Aminoff[7] as described by Greenberg et al.[5] The amount of sialic acid in the suspending fluid of untreated platelets was subtracted before the percent release or loss of sialic acid was calculated.

RESULTS

Effect of ADP-Induced Aggregation on Platelet Sialic Acid

Suspensions of washed rabbit platelets in Tyrode solution containing apyrase and 0.35% albumin aggregated strongly in response to ADP in the concentration range of 10 μM–1 mM without the release of detectable amounts of 14C-serotonin. During this reaction there was no increase in the amount of sialic acid in the platelet suspending medium (Table 1). The assay used would have detected the loss of 3% or more of the total sialic acid from the platelets. Total sialic acid of rabbit platelets was 27.7 ± 1.0 nmol/10⁹ platelets (mean ± SEM).

When washed human platelets were resuspended in Tyrode solution containing apyrase and 0.35% albumin, 0.1 or 1 mM ADP in the presence of fibrinogen (0.3 mg/ml) caused aggregation followed by deaggregation. Less than 1% release of either 14C-serotonin or sialic-acid-containing material was detected with platelets from 7 different subjects (Table 1). In a medium without calcium, 1 mM ADP induced the release of 42.5 ± 3.0% of the 14C-serotonin but only 3.4 ± 0.9% of the total platelet sialic acid appeared in the suspending medium (Table 1, mean values ± SEM from 5 subjects). Total sialic acid of human platelets was 34.5 ± 2.3 nmole/10⁹ platelets (mean ± SEM).

Effect of Thrombin on Human Platelet Sialic Acid

With platelets from 4 subjects, thrombin (5 U/ml) caused the release of 20.6 ± 7.4% of total platelet

Table 1. Effect of ADP-Induced Aggregation on the Release of 14C-Serotonin and on the Amount of Sialic Acid in the Suspending Fluid of Washed Rabbit or Human Platelets

<table>
<thead>
<tr>
<th>Species</th>
<th>Suspending Medium</th>
<th>No. of Experiments</th>
<th>ADP</th>
<th>14C-serotonin Released (% of Total)</th>
<th>Sialic Acid Released or Lost (% of Total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit</td>
<td>Tyrode-albumin</td>
<td>3</td>
<td>0.1 μM</td>
<td>0.1 ± 0.1*</td>
<td>0.2 ± 0.2*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>1.0 μM</td>
<td>0.96 ± 0.42*</td>
<td>0.6 ± 0.4*</td>
</tr>
<tr>
<td>Human</td>
<td>Tyrode-albumin</td>
<td>7</td>
<td>0.1 μM</td>
<td>0.1 ± 0.1*</td>
<td>0.2 ± 0.2*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>1.0 μM</td>
<td>0.96 ± 0.42*</td>
<td>0.6 ± 0.4*</td>
</tr>
<tr>
<td></td>
<td>Calcium-free</td>
<td>7</td>
<td>0.1 μM</td>
<td>0.1 ± 0.1*</td>
<td>0.2 ± 0.2*</td>
</tr>
<tr>
<td></td>
<td>Tyrode-albumin</td>
<td>5</td>
<td>1.0 μM</td>
<td>42.5 ± 3.0*</td>
<td>3.4 ± 0.9*</td>
</tr>
</tbody>
</table>

Platelet counts: Rabbit platelets 500,000/cu mm; human platelets, 700,000/cu mm.

Human fibrinogen (final concentration 0.3 mg/ml) was added to the human platelet suspensions before ADP. Samples for measurement of 14C-serotonin and loss of sialic acid were taken 3 min after the addition of ADP. In the calcium-free medium, all these human platelet preparations released 14C-serotonin, indicating that the second phase of ADP-induced aggregation had occurred.

*Mean ± SEM.
sialic acid while inducing the release of 90.0% ± 3.4% of 14C-serotonin (means ± SEM).

**Effect of Neuraminidase on Human Platelet Sialic Acid**

Neuraminidase (20 U/ml) removed 42.3% ± 1.9% (mean ± SEM of 4 subjects) of total human platelet sialic acid upon incubation for 60 min at 37°C (platelet count 10⁶/cu mm). Increasing the concentration of neuraminidase did not result in the removal of additional sialic acid. No 14C-serotonin was released during the incubation.

**Platelet Survival**

When 51Cr-labeled rabbit platelets were aggregated with 250 μM ADP, recovered as disc-shaped platelets, and injected into rabbits, the mean percentage in the circulation 1 hr after injection was 91.0% ± 3.6% compared with 87.6% ± 2.9% of the control platelets (means ± SEM of 9 rabbits in each group). The platelet survival curves based on the amounts of 51Cr in the circulation 1 hr were very similar (Fig. 1). The survival of ADP-treated platelets that were in the circulation at 1 hr was 62.0 ± 5.8 hr (mean ± SEM of 9 experiments). This was not significantly different from the survival of control platelets that had been subjected to the same washing and resuspending procedures (65.7 ± 7.2 hr).

**DISCUSSION**

Our values for total sialic acid of human platelets (34.5 ± 2.3 nmole/10⁹ platelets) are in accord with those of Marcus and colleagues ⁸,¹⁹ and Baenziger et al.⁶ They are, however, lower than the values of approximately 60 nmole/10⁹ platelets reported by some other investigators.¹,²,³,¹⁰,¹¹ Wu and Ku⁴ show values of 39 and approximately 50 nmole/10⁹ platelets. A wide range (40–90 nmole/10⁹ platelets) was reported by Peerschke and Zucker.²

The results of the present study indicate that the sialic acid on the surface of rabbit or human platelets is not lost when platelets change shape and aggregate in response to ADP and then deaggregate without release of granule contents. When human platelets are washed and resuspended in a medium containing no added Ca⁺⁺, ADP-induced aggregation occurs in two phases, the second of which is accompanied by the release of amine storage granule contents.¹ Although the second phase of aggregation was accompanied by more than 40% release of 14C-serotonin in our experiments, less than 4% of the total sialic-acid-containing material appeared in the suspending medium. Wu and Ku⁴ reported that when human platelets were exposed to ADP in a medium without calcium, the sialic acid available for cleavage by neuraminidase increased from 27 ± 5 to 30 ± 5 nmole/10⁹ platelets. In their medium, release of granule contents probably occurred during ADP-induced aggregation, and if so, may have been responsible for this slight increase in sialic acid that could be acted upon by neuraminidase. Apparently they did not resuspend their platelets in fresh medium before making their determinations. Our findings are difficult to reconcile with the 21.5% decrease in sialic acid content that Bunting et al.¹ observed during ADP-induced aggregation and attributed to a generalized loss of surface sialic acid or glycoprotein. Our results are in accord, however, with those of Peerschke and Zucker² who showed no change in sialic acid removable by neuraminidase when platelets were induced to change shape by ADP.

Most investigators have observed that approximately 40%–50% of the sialic acid of intact platelets is susceptible to neuraminidase and is therefore probably exposed on the platelet surface¹⁻⁵ (present results). However, values as high as 60% have been reported.²²,²³ The remainder is probably distributed among several locations: in the storage granules; on the surface if there is sialic acid that is not susceptible
to cleavage by neuraminidase; and in other platelet constituents. Several investigators have shown that material containing sialic acid is released when platelets are stimulated by thrombin.\textsuperscript{35-38} We observed 7\%–41\% (mean 20.6\% ± 7.4\%) release of sialic acid by thrombin under conditions in which 81\%–96\% of the \textsuperscript{14}C-serotonin was released. Peerschke and Zucker\textsuperscript{3} found that 30\% and 43\% of the total sialic acid was released by thrombin in the two subjects they studied. Earlier investigators reported 12\%, 14\%–18\%, and 27\% of the total sialic acid of human platelets to be releasable by ADP.\textsuperscript{6-8} At least some of this is probably the "thrombin-sensitive protein" described by Baenziger et al.\textsuperscript{6} and platelet fibrinogen.\textsuperscript{24}

Sialic-acid-containing material is less readily released than \textsuperscript{14}C-serotonin. When human platelets labeled with \textsuperscript{14}C-serotonin are aggregated by ADP in a medium containing a low concentration of ionized calcium, 40\%–60\% of the \textsuperscript{14}C-serotonin is released\textsuperscript{25-27} (present results). In contrast, we observed less than 4\% release of total sialic acid from human platelets aggregated by ADP under these conditions. Thus, little of the releasable sialic-acid-containing material in platelets is likely to be in the dense granules or in the α-granules whose contents are released somewhat more readily than those of the dense granules.\textsuperscript{28}

The observation that the survival of ADP-treated rabbit platelets in the circulation was not significantly shortened is in accord with the finding that their surface sialic acid must be essentially unchanged. In earlier experiments, we found that if as little as 8\% of the surface sialic acid had been removed with neuraminidase, platelet survival was shortened.\textsuperscript{5,9} Removal of glycopeptides from membrane glycoproteins with proteolytic enzymes also shortens platelet survival.\textsuperscript{9}

These observations with platelets aggregated by ADP in vitro are in keeping with the results of earlier experiments with thrombin-treated platelets.\textsuperscript{29} Rabbit platelets that were induced to release their granule contents by treatment with thrombin in vitro survived for a normal length of time when returned to the circulation.\textsuperscript{29} These platelets would have been exposed to released ADP. Thus, platelet survival is not significantly shortened by exposure to ADP in vitro and it seems unlikely that ADP causes the loss of platelet surface sialic acid.

REFERENCES


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