Effect of Antibody Binding and Agglutination on Human Platelet Glycolysis: Comparison with Thrombin and Epinephrine

By Simon Karpatkin and Gregory W. Siskind

The presence of human platelet antibodies and their deleterious effect on platelet survival has been well established. Although the sites for the sequestration and destruction of the antibody-coated platelets are known, the detailed mechanism for this destruction remains to be elucidated. A direct effect of antibody coating and agglutination on platelet metabolic activity was considered a possible mechanism of decreased platelet viability. Since the major portion of platelet energy metabolism is derived from glycolysis, the effect of platelet antibody on glycolytic parameters was investigated. A metabolically active washed platelet system free of variables introduced by the presence of plasma was used for these experiments. It is recognized that the behavior of platelets incubated in vitro with heterologous antibody may be different from their behavior with autologous antibody in vivo. Nevertheless, it was felt that these observations made with heterologous antibody might provide some understanding of the action of platelet antibodies in human disease. It was also considered of theoretical interest to compare glycolytic changes initiated by platelet antibody with glycolytic changes mediated by known physiologic initiators of platelet agglutination and contraction—thrombin and epinephrine. Glucose uptake, lactate production, ATP, glucose-6-phosphate (G-6-P) and intracellular glucose levels were measured in the presence and absence of rabbit antihuman platelet antibody.

Methods

Human platelet-rich plasma collected in ACD solution was obtained from the American Red Cross and The New York Blood Center within 2 or 3 hours after phlebotomy. It was kept over ice during delivery and handled in nonglass containers: Fenwal triple pack plastic bags, cellulose nitrate centrifuge tubes and plastic pipettes. The platelets were separated from their plasma by differential centrifugation in an International PR 2 Centrifuge at 5°C. The platelet-rich plasma was centrifuged at 350 g to remove red blood cells. The supernatant platelet suspension was decanted and centrifuged at 2250 g for 25 minutes. The platelet button obtained was suspended in 40 times its volume of a modified Human Ringer solution: 2 mM KC1, 107 mM NaCl, 20 mM NaHCO3, 2 mM Na2SO4, 0.1 mM EDTA gassed with 5 per cent CO2 and 95 per cent O2 to a pH of 7.1. Platelets were from the Department of Medicine, New York University Medical Center, New York, N.Y. Supported by General Research Support Grant, NIH, Fr 5399-05, and U. S. Public Health Service Research Grant No. 5-RO1-AM8805. Drs. Karpatkin and Siskind are Career Scientists of The Health Research Council of the City of New York, Investigatorships I-459 and I-464.

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suspended by gentle aspiration and return of the Ringer solution with a plastic pipette. The suspension was resedimented for 10 minutes at 2250 g and again suspended in approximately 40 times its volume of Ringer solution. Platelets were washed twice in this manner, with reagents and platelet suspension kept at ice bucket temperature. Platelet clumping was not observed in the final platelet suspension. Contamination with other blood elements was negligible.

**Incubation Procedure:** Four ml. aliquots of the final platelet suspension were employed for in vitro incubations. The packed cell volumes of the platelet suspensions, as measured in a microhematocrit tube, were 3-6 per cent (1 ml. packed platelets was equivalent to $1 \times 10^{10}$ platelets). Measurements were performed in duplicate on two different dilutions of the platelet suspensions and agreed within 5-10 per cent. Incubations were performed at 37°C for 1 hour under 5 per cent CO₂ and 95 per cent O₂ in tightly stoppered 40 ml. Sorvall cellulose nitrate centrifuge tubes in a gyratory New Brunswick Shaker at 25 r.p.m. Experiments were performed with pooled platelets from 3-4 units of blood. Appropriate dilutions of rabbit antihuman platelet antibody or antibody fragments (see below) were added to incubation mixtures. Control tubes contained normal rabbit gamma globulin or fragments prepared from normal rabbit gamma globulin in equivalent amounts based upon protein concentration estimated from the 280 nm absorbancy.

**Preparation of Rabbit antihuman Platelet Antibody:** Rabbits were injected in the footpads with washed human platelets emulsified in complete Freund’s adjuvants and boosted with intraperitoneal and subcutaneous injections of washed human platelets at weekly intervals for 9 weeks. One week after the last boost, the animals were bled and their sera collected. A globulin fraction was prepared by precipitation at 50 per cent cold saturated (NH₄)₂SO₄. The precipitated globulin was dissolved in a volume of distilled water approximately equal to that of the original serum sample and dialyzed against phosphate buffered saline (PBS: 0.15 M NaCl, 0.01 M potassium phosphate buffer, pH 7.5). The antibody caused rapid agglutination of platelets at a dilution of 1:400 and produced no macroscopic agglutination at a dilution of 1:2000.

**Preparation of Univalent Antibody Fragments:** Univalent antibody fragments (Fab) were prepared by reduction of the divalent antibody fragments (Fab₂) obtained following pepsin digestion of the intact antibody. Serum globulin preparations were incubated for 18 hours at 37°C with pepsin (equivalent in weight to 1 per cent of the protein present) in 0.1 M acetate buffer, pH 4.0. The digestion was terminated by bringing the pH to 8.0 with solid Na₂CO₃. The digested globulin was precipitated at 18 per cent Na₂SO₄, collected by centrifugation, dissolved in distilled water and dialyzed against PBS. Reduction of Fab fragments with 0.01 M 2-mercaptoethylamine was accomplished by incubation for 2 hours at 37°C in 0.1 M acetate buffer, pH 5.0. Free disulfide bonds were blocked by addition of a threefold molar excess of iodoacetamide. The product was extensively dialyzed in the cold against PBS before use. Binding of Fab fragments to platelets was established by the ability of the antibody-coated platelets to be agglutinated by a goat antirabbit globulin antiserum.

**Enzymatic Measurements and Extraction of Platelets:** This was performed as previously described. Briefly, the supernatant Ringer solution and platelet pellet were extracted with perchloric acid and the perchlorate ion was removed with KOH. Suitably diluted aliquots of the neutralized solutions were employed to measure extracellular lactate and glucose, and intracellular lactate, ATP, G-6-P, and glucose. Enzymatic methods employed nucleotide absorbancy changes at 363 and 340 nm for oxidation of acetyl-DPN and TPN, respectively. Lactate was measured by a minor modification of the Lowry procedure, employing acetyl-DPN and beef heart lactic dehydrogenase. ATP was measured by classic procedures coupling the hexokinase reaction to the G-6-P dehydrogenase reaction. Glucose was measured by the use of hexokinase and ATP coupled to the G-6-P dehydrogenase reaction. G-6-P was measured with the use of G-6-P dehydrogenase. Measurements are expressed as µmol/ml packed platelets.

**Extracellular Space Measurements:** To measure intracellular glucose it was necessary to determine the extracellular space in the platelet pellet so that extracellular glucose entrapped in the platelet pellet could be subtracted from the total pellet glucose. The extracellular space was measured with inulin. Since platelet tissue extract and glucose
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interfere with the color development employed in this assay, it was necessary to subtract
the color development of control pellets which were not exposed to inulin or glucose
during the incubation. Pellets were centrifuged at 3000 g for 10 minutes, extracted with
perchloric acid and neutralized with KOH as described previously. Under these condi-
tions the extracellular space represented 9 per cent by volume of the platelet pellet.

Materials: All chemicals employed were reagent grade. Glucose-6-P dehydrogenase
type V, crystalline hexokinase, ATP, TPN and acetyl-DPN were obtained from Sigma
Chemical Co., St. Louis, Mo. Inulin was obtained from Warner-Chilcott, Morris Plains,
N. J. Beef heart lactic dehydrogenase and pepsin were obtained from Worthington Bio-
chemical Corp., Freehold, N. J. 2-Mercaptoethylamine hydrochloride was obtained from
Cal Biochem, Los Angeles, Calif. Iodoacetamide was obtained from Mam Research, N. Y.

RESULTS

Platelets were incubated at 37 C. for 1 hour in the presence or absence of
1:20 dilution of rabbit antihuman platelet antibody. Under these conditions
agglutination of platelets occurred only with antibody (within 3 minutes). Table 1 shows the averages of 5 to 7 independent determinations of lactate pro-
duction, glucose uptake, ATP and G-6-P levels. In the absence of glucose, lac-
tate production increased 10.8 μm./ml./hr. or 42 per cent, ATP declined to 44
per cent of its control value, and G-6-P remained unaltered. In the presence of
5 mM glucose, lactate production increased 10.8 μm./ml./hr. or 31 per cent,
and glucose uptake increased 9.2 μm./ml./hr. or 47 per cent. ATP levels de-
dined to 44 per cent of their control values and G-6-P levels remained un-
changed. After subtracting extracellular glucose entrapped in the pellet from
total pellet glucose, it was clear that free glucose was not present in control
or antibody-treated platelets (4 experiments). Similar changes in glycolytic
parameters were obtained with antibody dilutions of 1:100 and 1:400. At
antibody dilutions of 1:2000 and 1:10,000 neither platelet agglutination nor
changes in glycolytic parameters were noted. Thus, changes in energy met-
abolism were detected only with antibody concentrations which resulted in
agglutination of platelets.

Incubations with univalent antibody fragments (Fab) prepared from rabbit
antihuman platelet antibody were similarly performed in the presence and
absence of glucose. Control platelets were incubated with Fab fragments pre-
pared from normal rabbit gamma globulin at equal protein concentration. Proof of binding of Fab fragments to human platelets was established by the
following experiment. Fab fragments were incubated with platelets for 15
minutes at 37 C. The platelets were then sedimented at 4000 g, 5 C., and the
supernatant removed. The platelet pellets were resuspended in 1 ml. of human
Ringer solution. To this suspension, 0.1 ml. of goat antirabbit globulin anti-
serum (2 mg. antibody/ml.) was added and the suspension incubated at 37 C.
Platelets which had been exposed to Fab fragments prepared from antiplatelet
antibody agglutinated promptly upon addition of goat antirabbit globulin
antibody. Platelets exposed to Fab fragments obtained from normal rabbit
globulin did not demonstrate agglutination upon addition of antirabbit globu-
lin antibody. Table 1 indicates the effect of binding of antibody fragments
upon platelet glycolysis after incubation at 37 C. for 1 hour. No changes
were noted in lactate production, glucose uptake or ATP levels.

Table 2 compares the effect of other initiators of platelet agglutination/con-
Table 1.—Effect of Platelet Antibody Binding and Agglutination on Glycolysis

<table>
<thead>
<tr>
<th>5 mM Glucose</th>
<th>Lactate Production</th>
<th>Glucose Uptake</th>
<th>Adenosine Triphosphate</th>
<th>Glucose-6-Phosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (7)</td>
<td>25.5 ± 1.4</td>
<td>34.5 ± 1.8</td>
<td>17.7 ± 2.0</td>
<td>1.98 ± .12</td>
</tr>
<tr>
<td>Platelet</td>
<td>30.3 ± 1.1</td>
<td>43.3 ± 1.6</td>
<td>26.9 ± 2.4</td>
<td>0.88 ± .13</td>
</tr>
<tr>
<td>Antibody (7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (5)</td>
<td>22.7 ± 1.5</td>
<td>30.3 ± 2.1</td>
<td>16.1 ± 1.9</td>
<td>1.84 ± .16</td>
</tr>
<tr>
<td>Fab (5)</td>
<td>23.4 ± 1.4</td>
<td>29.7 ± 1.0</td>
<td>16.2 ± 2.1</td>
<td>1.80 ± .16</td>
</tr>
</tbody>
</table>

1 μm/ml/hr.
2 μm/ml. at the end of one hour.
3 Number of experiments is given in brackets.
4 Standard error of the mean.
5 Univalent antiplatelet antibody fragment (see Methods).

Table 2.—Comparison of Glycolytic Changes Following Platelet Agglutination and Contraction with Different Agents

<table>
<thead>
<tr>
<th>5 mM Glucose</th>
<th>Glucose Uptake</th>
<th>Lactate Production</th>
<th>ATP Level</th>
<th>Glucose-6-P Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (8)</td>
<td>18.5</td>
<td>21.7</td>
<td>36.6</td>
<td>1.98</td>
</tr>
<tr>
<td>μm/ml/hr.</td>
<td></td>
<td></td>
<td></td>
<td>1.95</td>
</tr>
<tr>
<td>Thrombin (6)</td>
<td>15.6</td>
<td>8.0</td>
<td>18.7</td>
<td>-1.36</td>
</tr>
<tr>
<td>Epinephrine (6)</td>
<td>9.3</td>
<td>8.1</td>
<td>16.4</td>
<td>-0.56</td>
</tr>
<tr>
<td>Platelet Ab</td>
<td>9.2</td>
<td>10.8</td>
<td>10.8</td>
<td>-1.10</td>
</tr>
</tbody>
</table>

1 Values given are ± increment from control at the end of one hour: μm/ml/hr. for lactate and glucose, μm/ml. for ATP and G-6-P. For further details, see ref. 8.
2 Control values obtained after aerobically incubating platelets for 1 hour at 37 C.
3 Thrombin, 1 unit/ml.
4 1 × 10^-6 M epinephrine plus 1 × 10^-6 M ascorbic acid.
traction with the effect of antiplatelet antibody on platelet glycolysis. The increment, ± control values, of glucose uptake, lactate production, ATP and G-6-P levels are depicted at the end of 1 hour. It should be noted that platelet antibody behaved differently from thrombin and epinephrine in that increased glucose uptake was not accompanied by a parallel increase in lactate production. In the absence of glucose, platelet antibody, epinephrine and thrombin increased lactate production (glycogenolysis) and decreased ATP levels. In the absence of these agents, glucose alone increases lactate production (see controls, Table 2). This glucose-mediated increment in lactate production persisted in the presence of all 3 agents. However, in the presence of glucose a further increase in lactate production resulted with thrombin and epinephrine, but not with platelet antibody.

**DISCUSSION**

There are a few reports in the literature on the effect of antiplatelet antibody upon platelet function. Pachter et al.,12 working with macroglobulins, reported a decrease in platelet factor 3 release and thromboplastin generation following coating by this material, although clot retraction remained normal. Gorstein et al.13 reported that antiplatelet antibody inhibited clot retraction and thromboplastin generation. Bridges et al.,14 employing rabbit antihuman platelet antibody, described a decrease in serotonin uptake by platelets following exposure to both agglutinating and nonagglutinating concentrations of platelet antibody.

In our study, concentrations of antiplatelet antibody insufficient to cause agglutination had no measurable effect on platelet glycolysis. Similarly, univalent antibody fragments, although capable of binding to platelets, had no effect on platelet glycolysis. These results suggest that simple antibody binding does not account for the glycolytic depletion of antibody-coated platelets.

Platelet agglutination, however, was associated with a significant burst of glycogenolytic activity and a net loss of ATP. Since this decrease in ATP was associated with an increase in lactate production, increased ATP utilization must be postulated to occur and possibly increased ATPase activity. The expenditure of ATP following antibody agglutination is probably not a major mechanism for platelet destruction, because once agglutinated by antibody, platelets are rapidly cleared by the reticuloendothelial system. It is recognized that the behavior of platelets incubated in vitro with heterologous antibody or Fab fragments may be different from their behavior with autologous antibody in vivo.

It is of interest that glucose uptake was not impeded by antibody binding or agglutination. Accordingly, simple coating of platelets by protein is insufficient to inhibit glucose transport sites. On the contrary, glucose uptake increased appreciably following agglutination. Comparisons with the effect of other initiators of platelet agglutination/contraction suggests that the insult of agglutination/contraction results in a burst of glycolytic activity and expenditure of ATP. All agents behaved in a similar fashion. Elevated G-6-P levels in the presence of epinephrine suggest that epinephrine activates phosphoryl-
ase, with enzymes removing C-6-P becoming rate limiting. Similar findings have been reported with skeletal muscle.\textsuperscript{15}

Of interest are the observations on glycolytic activation induced by platelet antibody. In the absence of glucose, platelet antibody resulted in an increase in lactate production (glycogenolysis). In the presence of glucose, increased glucose uptake was not accompanied by a parallel increase in lactate production, as was the case for thrombin or epinephrine. However, the normal glucose-mediated lactate production persisted (Table 1), and free glucose did not accumulate intracellularly. Consequently, one might postulate the following: In the absence of glucose all 3 agents activate lactate production (glycogenolysis). In the presence of glucose all 3 agents activate the uptake of glucose and consequent production of glucose-6-P (increased hexokinase activity). Platelet antibody differs from thrombin and epinephrine in that it activates a glucose-requiring nonglycolytic pathway, perhaps the hexosemonophosphate shunt or the Krebs’ cycle. It is conceivable that all 3 agents activate the Embden-Meyerhof (glycolytic) pathway but that platelet antibody shunts substrate to the Krebs’ cycle enzymes rather than to lactate.

The universal glycogenolytic response of platelets to agglutination/contraction whether mediated by thrombin, epinephrine or platelet antibody, suggest that activation of the glycolytic chain and ATP expenditure may be a result of the cellular reorganization inherent in this process. This is not to imply that nonagglutinating concentrations of these effector agents are incapable of activating glycogenolysis. For example, Corn\textsuperscript{16} has reported increased lactate production with nonagglutinating concentrations of thrombin.

**SUMMARY**

Freshly collected human platelets were washed in a modified human Ringer solution, pH 7.1, and aerobically incubated in the same media for 1 hour in the presence or absence of glucose. The effect of rabbit antihuman platelet antibody or univalent rabbit antihuman platelet antibody fragments (Fab) on platelet glycolysis was determined. Although ATP expenditure and glycogenolytic depletion were noted following platelet agglutination by antibody, these changes were not considered to be of major importance in the in vivo destruction of platelets. Univalent fragments were shown to bind to platelets without causing platelet agglutination or any detectable change in the glycolytic parameters. In contrast, intact platelet antibody resulted in platelet agglutination which was associated with an increase in lactate production and a decrease in ATP levels when platelets were incubated in the absence of glucose. Glucose-6-P levels did not change. When platelets were incubated in the presence of glucose, glucose uptake increased, ATP levels declined and glucose-6-P levels were unchanged. However, the increased glucose uptake was not accompanied by a parallel increase in lactate production as in the case with thrombin or epinephrine-induced agglutination of platelets. It is postulated that platelet antibody activates a glucose-requiring nonglycolytic pathway, perhaps the hexosemonophosphate shunt or the Krebs’ cycle.

**SUMMARIO IN INTERLINGUA**

Frescamente colligite plachettas human esseva lavate in un modificate solution de Ringer
human, a pH 7.1, e incubate aerobiemente in le mesme medio durante un hora in le presentia o in le absentia de glucosa. Le effecto de anticorpore de conilio anti plachettas human o de univalente fragmentos de anticorpore de conilio anti plachettas human super le glycolyse del plachettas esseva determinate. Ben que un expendition de ATP e un depletion glycogenolyte esseva notate post le agglutination anticorpora del plachettas, iste alterationes non esseva reguardate como particularmente importante pro le destruction in vivo de plachettas. Esseva monstrate que fragmentos univalente se ligava con plachettas sin causar un agglutination plachettal o unle detegibile alteration in le parametos glycolytic. Per contrasto, intacte anticorpore anti plachettas resultava in un agglutination plachettal associate con un augmento in le production de lactato e un declino in le nivellos de ATP quando le plachettas esseva incubate in le presentia de glucosa. Le nivellos de glucosa-6-phosphato non esseva afficite. Quando le plachettas esseva incubate in le absentia de glucosa, le aceptation de glucosa montava, le nivellos de glucosa-6-phosphato remaneva sin alteration. Tamen, le augmentate aceptation de glucosa non esseva accompaniate per un augmento parallel in le production de lactato, como il es le caso in agglutination plachettal induce con thrombina o epinephrina. Es postulate que anticorpore plachettal effectua un activation de un circuito nonglycolytic que require glucosa, possibilemente le derivation hexoso-monophosphatic o le ciclo de Krebs.

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