Effect of Heterologous Antiplatelet Antibody on Human Platelets: A New Pathway to Platelet Alteration

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Heterologous monomeric IgG anti–human platelet antibody mediates platelet aggregation and release of $^{14}$C-serotonin from human platelets. The reaction is independent of complement and thrombin and is not inhibited by acetylsalicylic acid. Studies are presented showing that when adenosine diphosphate (ADP) was removed by apyrase, creatine phosphokinase, or pyruvate kinase, antibody-induced aggregation and serotonin release were only inhibited 50% and 35%, respectively. Inhibition of both aerobic metabolism with antimycin A (5.0 μg/ml) and anaerobic glycolysis with 2-deoxyglucose (10 mM) completely prevented ADP and thrombin-induced aggregation and release. However, inhibition of antibody-mediated aggregation and release was incomplete at high concentrations of antibody. Dibutyryl cyclic adenosine 3',5'-monophosphate (cAMP) inhibited antibody-induced aggregation and release in a dose-related fashion, but required ten-fold the concentration necessary to inhibit the effect of ADP. Prostaglandin E$_1$, which stimulates adenylate cyclase to form cAMP, inhibited the antibody effects 50% at 50-fold the concentration required for equivalent inhibition of aggregation and release by epinephrine. The phosphodiesterase inhibitor SH869 (2 μM) gave similar results. These data suggest that heterologous antibody (IgG)-induced platelet aggregation and release are mediated in part by a mechanism independent of extracellular ADP and in part independent of metabolic energy. The high concentration of cAMP required for inhibition suggests a pathway distinct from that mediated by ADP, epinephrine, or thrombin.

HETEROLOGOUS (rabbit) IgG anti human platelet antibody has been shown to mediate platelet aggregation and $^{14}$C-serotonin release as well as to increase the availability of platelet acid phosphatase. The effect of rabbit antiplatelet antibody on platelets proceeds in the absence of complement and is mediated by monomeric IgG. The reaction does not require thrombin and is inhibited by 2 mM EDTA. The nonsteroidal antiinflammatory agent, acetylsalicylic acid, however, does not inhibit the antibody-induced effects, indicating that endogenous platelet prostaglandin synthesis is not involved. In order to delineate further the mechanism(s) of antibody-induced platelet aggregation and release, we have studied their dependence on metabolic energy, their requirement for adenosine diphosphate (ADP), and their involvement with the cyclic adenosine 3',5'-monophosphate (cAMP) system.
MATERIALS AND METHODS

ADP, epinephrine bitartrate, creatine phosphokinase type I, disodium creatine phosphate, pyruvate kinase type III, trisodium phosphoenol pyruvate, 2-deoxy-D-glucose, and dibutyryl cAMP were obtained from the Sigma Chemical Co., St. Louis, Mo.; prostaglandin E1 (PGE1) was kindly supplied by J. Pike, Upjohn, Kalamazoo, Mich.; and SH869 was purchased from Pharma-Research, Montreal, Quebec, Canada. Potato apyrase was a gift from Dr. S. Niewiarowski, Temple University, Philadelphia, Pa. Highly purified human α thrombin was a gift from Dr. John Fenton, Division of Laboratories and Research, N.Y. State Dept. of Health, Albany, N.Y..

Platelet-rich Plasma (PRP)

Human blood from normal volunteers was collected into anticoagulant solution (1 part 3.8% sodium citrate to 9 parts whole blood) and centrifuged at 23°C for 10 min at 170 g; the supernatant PRP was removed by aspiration and used for the studies. Whole blood and plasma were only exposed to plastic surfaces. The platelet count of PRP varied from 200,000 to 350,000/μl. The white blood cell and red blood cell counts were 3 and 2/100,000 platelets, respectively, as determined by light microscopy. All studies employed PRP, except for those involving ADP depletion or thrombin. Experiments with thrombin were performed with both PRP and gel-filtered platelets. Because coagulation occurred in PRP with thrombin, only the thrombin experiments with gel-filtered platelets are reported.

Platelet-poor Plasma (PPP)

Blood was collected as for PRP and centrifuged at 23°C at 12,000 g for 4 min in an Eppendorf centrifuge (Brinkman Instruments, Westbury, N.Y.), and the supernatant PPP was harvested. The platelet count was less than 1000 platelets/μl.

Gel-filtered Platelets

Gel-filtered platelets were employed for the studies involving ADP utilizing enzymes to avoid the influence of possible plasma inhibitors or competing enzymes. Platelets were filtered through Sepharose 2B using a modification of the method of Tangen et al.3 as previously described.1 A modified Tyrode’s buffer, pH 7.4, containing 0.3%, crystalline human serum albumin (Pentex, Miles Laboratories, Kankakee, Ill.), dextrose 0.1%, Mg2+ (2 mM) and no added calcium was employed. The final concentration of calcium as measured by atomic absorption spectroscopy was 38 μM. Fractions containing platelets appeared at the void volume of the column and were pooled so as to contain 150,000-250,000 platelets/μl. There were no detectable leukocytes and only 1 erythrocyte/100,000 platelets. Prior to each experiment (except those employing thrombin), 3 mg/ml human 95% clottable fibrinogen (Kabi, Stockholm, Sweden) was added to the gel-filtered platelet suspension.

Platelet Aggregation

Platelet aggregation was carried out as previously described4 in the Chronolog aggregometer. The transmittance of PRP was arbitrarily set at 10, and that of PPP was set at 90. The percentage aggregation was defined as the percentage change in transmittance between PPP and PRP. For aggregation and serotonin release studies with ADP a single concentration was employed for each experiment. This concentration was the lowest necessary for maximal aggregation.

Platelet 14C-Serotonin Release

The release of 14C-serotonin from human platelets was assessed by a modification of the method of Jerushalmi and Zucker.5 PRP was incubated with 8 μCi of 14C-serotonin for 30 min at 37°C, at which time 80%–90% of the radioactivity was bound to the platelets. The percentage released into the supernatant following platelet sedimentation at 12,000 g for 4 min was equal to

\[
\frac{\text{cpm in supernatant after incubation} - \text{cpm in supernatant prior to incubation}}{\text{total cpm in PRP} - \text{cpm in supernatant prior to incubation}} \times 100.
\]
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Platelet Lactic Dehydrogenase Loss

This determination was performed as previously described after incubation of the PRP with metabolic inhibitors (see below) with and without antibody. No interference with the assay by antimycin A or 2-deoxyglucose was detected at the concentrations employed.

Preparation of Rabbit Anti-Human Platelet Antisera

Human blood (60 ml) was collected into 2 ml of 5% ethyldinitrilotetraacetate (EDTA) in plastic tubes (Falcon Plastics, Oxnard, Calif.). PRP was prepared by sedimentation at 200 g at 23°C for 6 min and washed platelets were utilized to prepare rabbit anti-human platelet antisera as previously described. Although fresh rabbit antiplatelet antiserum contained approximately 500,000 hemolytically effective C1 molecules/ml, heating the antiserum at 58°C for 30 min destroyed 100% of the hemolytically effective rabbit C1.

Since normal platelets vary quantitatively in their response to antiplatelet antibody, in each experiment the dose response effect of the antiplatelet antibody on platelet aggregation was studied first. As described, rabbit antiplatelet antibody induces platelet aggregation and 14C-serotonin release in a dose-dependent fashion which achieves a maximum. It was therefore important to select a concentration of antiserum just sufficient for maximal platelet aggregation and 14C-serotonin release. In such a manner, inhibition of the antibody-induced effect could be readily detected. The dilution of antisera employed which fulfilled these criteria ranged from 1:16 to 1:64. All dilutions were prepared with the identical modified Tyrode's buffer used for gel-filtered platelets. In a select study, experiments with a low concentration of antibody (1:128) were performed. Lower concentrations of antibody (1:256) failed to elicit significant release and aggregation. In each experiment parallel controls with ADP or epinephrine were examined so as to establish the functional integrity of each platelet preparation before and after experiments with antiplatelet antibody and to employ the minimal amount of ADP and epinephrine necessary for a maximal response in the inhibition studies.

Isolation of the antiplatelet antibody activity by sequential anion exchange, cation exchange, and gel filtration chromatography revealed that all detectable activity resided in the IgG fraction corresponding to a molecular weight of 160,000.

Measurement of Antibody-induced Platelet Aggregation and Release

Two-tenths ml of an appropriate dilution of heat inactivated serum and 0.3 ml PRP or gel-filtered platelets were mixed and the reaction was begun by stirring in a Chronolog aggregometer at 37°C. Aggregation was measured as indicated below during a 10-min period. The tube was transferred to an aluminum block maintained at 37°C by circulating water and then stirred at the same speed as the aggregometer, 1200 rpm, for an additional 35 min. Following this incubation, the platelets were centrifuged at 12,000 g for 4 min and aliquots of the supernatant were withdrawn for measurement of 14C-serotonin. The 14C-serotonin release was calculated as described above. All experiments were controlled with nonimmune serum which gave <10% platelet 14C-serotonin release and <5% aggregation.

Effect of ADP Depletion on Antiplatelet Antibody Effect

Three-tenths ml of 14C-serotonin gel-filtered platelets were preincubated at 37°C for 1 min with 50 μl of apyrase (4500 U/ml), 30 μl of creatine phosphate (0.1 M), and 5 μl of creatine phosphokinase (945 U/ml), or with 10 μl of phosphoenolpyruvate (15 mM) and 5 μl pyruvate kinase (1000 U/ml). In each experiment 0.2 ml of either rabbit antiplatelet antibody or ADP in buffer were added and stirred at 1200 rpm at 37°C for 45 min and 15 min, respectively. The percentage platelet aggregation and 14C-serotonin release were then determined.

Requirements for Metabolic Energy

Three-tenths ml of 14C-serotonin platelets in PRP or gel-filtered platelets were preincubated for 30 min at 37°C with 10 μl of 2-deoxyglucose (500 mM) and/or 1 μl of antimycin A (2.5 mg/ml in ethanol). To determine if these conditions depleted the metabolic pool of adenosine triphosphate (ATP), 2 ml of gel-filtered platelets were extracted with a final concentration of 50%
ethanol containing 3.5 mM EDTA, and ATP was determined by the luciferase method.\textsuperscript{7} An Aminco-Bowman spectrofluorometers modified for dark injection was used. Prior to the addition of the metabolic inhibitors, the concentration of ATP in the gel-filtered platelets was 8.3 \(\mu\)moles/10\(^{11}\) platelets, and this level was unchanged after 30 min of incubation in the absence of inhibitors. In contrast, after incubation with 2-deoxyglucose and antimycin A for 30 min, ATP in the gel-filtered platelets fell to 4.9 \(\mu\)moles/10\(^{11}\) platelets, representing 58\% of the control value. Since almost 60\% of the platelet adenine nucleotides are stored in the subcellular granules\textsuperscript{8} and are not depleted by metabolic inhibitors, it appeared that the metabolic pool of ATP was essentially completely depleted. The concentration of ethanol (0.2\%) did not influence platelet aggregation or serotonin release. Two-tenths ml of rabbit antiplatelet antibody, ADP, or thrombin was then added as above and the percentage \(^{14}\)C-serotonin release and aggregation were determined.

**Studies on the Influence of cAMP**

Three-tenths ml of \(^{14}\)C-serotonin platelets in PRP were preincubated for 1 min at 37\(\degree\)C with increasing concentrations of PGE\(_1\) or the phosphodiesterase inhibitor SH869, or for 10 min at 37\(\degree\)C with dibutyryl cAMP. Two-tenths ml of rabbit antiplatelet antibody, epinephrine, or ADP was added and the percentage \(^{14}\)C-serotonin release and aggregation were determined as above. SH869 is a dipyradimole derivative and antiplatelet agent\textsuperscript{9} similar to dipyradimole in that it inhibits platelet phosphodiesterase.\textsuperscript{10}

**RESULTS**

In a previous study it was shown that the effect of IgG antiplatelet antibody on human platelets was not inhibited by acetylsalicylic acid.\textsuperscript{1} This observation supported the concept that platelet aggregation and release caused by heterologous antiplatelet antibody is mediated at least in part by a mechanism different from that initiated by ADP and epinephrine. To investigate further the relationship between the platelet release reaction induced by antibody and ADP, studies were performed to examine the requirement for ADP. Three reactions which deplete ADP were studied (Table 1). Apyrase, (450 U/ml),\textsuperscript{11} which converts ADP to AMP, almost completely inhibited ADP-induced aggregation and release. Creatine phosphokinase, which in the presence of creatine phosphate converts ADP to ATP,\textsuperscript{12} similarly inhibited ADP-induced aggregation and \(^{14}\)C-serotonin release almost completely. Pyruvate kinase, which in the presence of its substrate, phosphoenolpyruvate, also converts ADP to ATP,\textsuperscript{13} substantially inhibited the ADP effects as well. All three of these ADP-depleting reactions, however, only partially inhibited the antibody-induced platelet aggrega-

**Table 1. Effect of ADP Utilization on Antibody-induced Aggregation and Release**

(Percent Inhibition)

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Antibody Aggregation</th>
<th>(^{14})C-Serotonin Release</th>
<th>ADP Aggregation</th>
<th>(^{14})C-Serotonin Release</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apyrase (450 U/ml)</td>
<td>55</td>
<td>35</td>
<td>88</td>
<td>91</td>
</tr>
<tr>
<td>Creatine phosphate (10 mM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Creatine phosphokinase (10 U/ml)</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Phosphoenolpyruvate (0.3 mM)</td>
<td>38</td>
<td>40</td>
<td>67</td>
<td>87</td>
</tr>
<tr>
<td>Pyruvate kinase (10 U/ml)</td>
<td></td>
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</tr>
</tbody>
</table>

Each value represents the mean of two experiments. ADP (50 \(\mu\)M) stimulated 70\% platelet aggregation and 58\% \(^{14}\)C-serotonin release in the absence of inhibitors. Rabbit antiplatelet antibody (1:64 dilution) caused 60\% platelet aggregation and 59\% \(^{14}\)C-serotonin release.
Table 2. Effect of Metabolic Inhibitors on Antibody-induced Aggregation (Percent Inhibition)

<table>
<thead>
<tr>
<th>Antibody Dilution</th>
<th>ADP 0.2 NIH U/ml</th>
<th>Thrombin 0.1 NIH U/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:32</td>
<td>1:64</td>
<td>1:128</td>
</tr>
<tr>
<td>2-Deoxyglucose (10 mM)</td>
<td>9 ± 4</td>
<td>55 ± 6</td>
</tr>
<tr>
<td>Antimycin A (5 µg/ml)</td>
<td>6 ± 3</td>
<td>9 ± 3</td>
</tr>
<tr>
<td>2-Deoxyglucose (10 mM) + antimycin A (5 µg/ml)</td>
<td>33 ± 1</td>
<td>39 ± 2</td>
</tr>
</tbody>
</table>

The values represent the mean ± SEM of 3-5 replicate experiments. Platelet aggregation was 70% with (3-10 µM) ADP, 75% with 0.2 U/ml thrombin, and 72% with 0.1 U/ml thrombin. Antiplatelet antibody caused a mean of 75% aggregation at 1:64, 63% at 1:128, and 20% at 1:256 dilution. The experiments with antibody and ADP were performed with PRP and those with thrombin, with gel-filtered platelets.

The requirements for metabolic energy in the antibody-platelet interaction were explored next (Tables 2 and 3). Two agents, one that inhibits aerobic metabolism (antimycin A) and another that inhibits anaerobic metabolism (2-deoxyglucose), were employed. For each inhibitor dose-response curves were obtained. It was found that at 10 mM 2-deoxyglucose, ADP-induced 14C-serotonin release was 89% inhibited (Table 3) and the second phase of aggregation was eliminated, with aggregation inhibited 55% (Table 2). Doubling the concentration of 2-deoxyglucose did not increase the extent of inhibition. Antimycin A alone at 5 µg/ml (Tables 2 and 3) and at 10 µg/ml (not shown) did not appreciably inhibit serotonin release or aggregation induced by ADP. The combination of 2-deoxyglucose (10 mM) and antimycin A (5 µg/ml) inhibited ADP- and thrombin-induced aggregation (Table 2) and 14C-serotonin release (Table 3) almost completely, and completely depleted the ATP metabolic pool. A dose-response curve for thrombin showed 90% inhibition of release at half the concentration of antimycin A and deoxyglucose employed. Increasing the concentration of each inhibitor twofold did not augment the level of inhibition. Although the data reported for the experiments with thrombin were performed with gel filtered platelets, qualitatively similar results were obtained with thrombin and PRP. Concentrations of thrombin of 0.025-0.2 NIH U/ml gave similar results.

Table 3. Effect of Metabolic Inhibitors on Antibody-induced 14C-Serotonin Release (Percent Inhibition)

<table>
<thead>
<tr>
<th>Antibody Dilution</th>
<th>ADP 0.2 NIH U/ml</th>
<th>Thrombin 0.1 NIH U/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:32</td>
<td>1:64</td>
<td>1:128</td>
</tr>
<tr>
<td>2-Deoxyglucose (10 mM)</td>
<td>5 ± 2</td>
<td>89 ± 12</td>
</tr>
<tr>
<td>Antimycin A (5 µg/ml)</td>
<td>5 ± 4</td>
<td>5 ± 5</td>
</tr>
<tr>
<td>2-Deoxyglucose (10 mM) + antimycin A (5 µg/ml)</td>
<td>2 ± 1</td>
<td>63 ± 21</td>
</tr>
</tbody>
</table>

Each value represents the mean ± SEM of 3-5 replicate experiments. 14C-Serotonin release was a mean of 30% with (3-10 µM) ADP, 85% with 0.2 NIH U/ml thrombin, and 72% with 0.1 NIH U/ml of thrombin. Antiplatelet antibody caused a mean of 93% release at 1:64, and 86% release at 1:128 dilution. At an antibody concentration of 1:256 no serotonin release was observed. The experiments with antibody and ADP were performed with PRP and those with thrombin, with gel-filtered platelets.
Control experiments using platelets preincubated with metabolic inhibitors for 30 min at 37°C did not reveal any \(^{14}C\)-serotonin release after 45 min of incubation at 37°C in the absence of antibody, ADP, or thrombin. Antibody-induced serotonin release was not accompanied by platelet lysis, as judged by lactic dehydrogenase loss.\(^1\) In addition, the combination of antibody and metabolic inhibitors (37°C for 30 min) did not lead to platelet lysis as measured by lactic dehydrogenase loss.

The results with rabbit antiplatelet antibody in PRP were entirely dependent on antibody concentration. With high concentrations of antibody (1:64) almost no inhibition of \(^{14}C\)-serotonin release or aggregation was observed with either 2-deoxyglucose or antimycin A. Similarly, \(^{14}C\)-serotonin release was not inhibitable by the combination of these inhibitors but aggregation was inhibited 39%. Further studies showed that when platelets were preincubated with 2-deoxyglucose and antimycin A, the presence of 10 mM EDTA inhibited antibody-induced aggregation and release 75%. However, when the effect of a lower concentration of antibody (1:128) was examined, considerable inhibition of both aggregation (66%) and serotonin release (63%) was evident in the presence of inhibitors of both aerobic and anaerobic metabolism.

Similar results were obtained with isolated rabbit IgG antiplatelet antibody.\(^1\) In the presence of both inhibitors at the above concentrations no inhibition of serotonin release was found at high IgG antiplatelet antibody concentrations, while aggregation was more sensitive, being inhibited 35% (not shown). At lower concentrations of partially purified antibody, serotonin release was inhibited 43% and aggregation was inhibited 62%. In addition, similar results were observed in these experiments with 2-deoxyglucose and antimycin A when gel-filtered platelets were employed. Results obtained with gel-filtered platelets confirmed the capacity of antimycin A in combination with 2-deoxyglucose to inhibit platelet aggregation and serotonin release induced by the antiplatelet antibody.

The mechanism of the rabbit antiplatelet antibody effect was further pursued by investigating the consequence(s) of increasing the platelet cAMP level. Platelet cAMP was increased by three means: by the addition of PGE\(_1\), which increases the activity of adenylate cyclase in platelets;\(^4\) by the addition of SH869, which inhibits phosphodiesterase, an enzyme necessary for cAMP catabolism; and by the addition of exogenous dibutyryl cAMP, a lipid soluble derivative of cAMP. PGE\(_1\), inhibited the antibody-induced aggregation (Fig. 1A) and \(^{14}C\)-serotonin release (Fig. 1B) in a logarithmic dose-response fashion. The inhibition was not complete, however, reaching a limiting value of 60% - 70% (2.8 \(\mu M\)). In contrast, PGE\(_1\) inhibited platelet aggregation and serotonin release by epinephrine 90% at a 20-fold lower concentration (0.14 \(\mu M\)). Similarly, there was a 50-fold difference in the 50% inhibition point between epinephrine and antiplatelet antibody. The phosphodiesterase inhibitor SH869 also inhibited the antibody-induced effects, with approximately 40% inhibition at concentrations as high as 8 \(\mu M\) (Fig. 2). Inhibition of epinephrine-mediated \(^{14}C\)-serotonin release was complete at 1.0 \(\mu M\) and inhibition of aggregation was greater than 80% at 2.0 \(\mu M\).

Dibutyryl cAMP also inhibited antibody-mediated platelet aggregation and
14C-serotonin release (Fig. 3) in a dose-dependent fashion with total inhibition of serotonin release being achieved with 2.5 mM dibutyryl cAMP. Complete inhibition of the release reaction caused by ADP required only 0.25 mM. Dibutyryl cAMP also prevented antibody-induced platelet aggregation, but the concentration required for 50% inhibition was about 10-fold greater than that for ADP-initiated aggregation.

Since PGE₁ has been shown to raise intracellular cAMP levels and SH869 to inhibit cAMP degradation by phosphodiesterase, we examined the capacity of both agents to inhibit the antibody effect. Three different dose combinations were employed. The addition of PGE₁ (1.4–2.8 μM) to SH869 (4.0–8.0 μM) resulted in enhancement of inhibition from 50% with the single agent to 70%–100% with the combination of PGE₁ and SH869. The effect on aggregation was not increased.
**DISCUSSION**

We have studied the mechanism of action of heterologous anti-human platelet antibody on platelet aggregation and $^{14}$C-serotonin release. Previously we had observed that acetylsalicylic acid in vitro did not inhibit the capacity of antiplatelet antibody to aggregate human platelets and induce the release of platelet $^{14}$C-serotonin. We had also observed that the ingestion of ASA did not inhibit the effect of the rabbit antibody on these platelets. Since acetylsalicylic acid is known to inhibit the synthesis of endogenous prostaglandins, endoperoxide intermediates, and thromboxanes, this finding indicated that the antibody effect on platelets was not mediated through the prostaglandin forming pathway.

Acetylsalicylic acid is known to inhibit the second phase of platelet aggregation and the release reaction initiated by ADP and epinephrine in citrated PRP. Since platelet ADP release occurs coincidently with secondary aggregation, we have examined whether the removal of ADP modifies the antibody-induced effect. In these experiments, sufficient ADP was added to control samples to produce secondary aggregation and serotonin release. The marked inhibition of ADP-induced aggregation and release induced with 50 μM ADP with the three ADP-utilizing enzyme systems studied indicates a sufficient capacity and velocity to transform any endogenous ADP released during antibody-induced alteration (Table 1). The observation that only partial inhibition of antiplatelet antibody was achieved suggests the existence of an ADP-independent pathway. It is conceivable, however, that antibody causes a marked increase in the sensitivity of the platelet to trace concentrations of ADP. Previously it had been shown that heterologous antiplatelet antiserum causes the release of platelet nucleotides. The partial inhibition achieved by ADP depletion supports the involvement of ADP release, but also suggests that another mechanism may be responsible for the antibody-mediated release reaction.

In agreement with Mürer, we observed that 2-deoxyglucose, which affects anaerobic metabolism by inhibiting the conversion of glucose-6-phosphate to fructose-6-phosphate by phosphoglucone isomerase, substantially inhibited ADP-induced $^{14}$C-serotonin release and secondary aggregation (Tables 2 and 3). In contrast, antimycin A, which inhibits aerobic metabolism by interfering with the transformation of cytochrome B to cytochrome C, did not inhibit ADP-induced release and aggregation. The combination of 2-deoxyglucose with antimycin A resulted in complete inhibition of ADP-induced primary ag-
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Aggregation as well, indicating an absolute requirement for metabolic energy in this reaction. The concentrations employed were also sufficient to inhibit completely thrombin-induced aggregation and release and completely depleted the ATP metabolic pool. In contrast, inhibition of both aerobic and anaerobic metabolism with antimycin A and 2-deoxyglucose, failed to alter significantly the antibody-induced release reaction at high antibody concentration. Thus, when the ratio of antibody to platelets was sufficient, release was partially independent of metabolic energy.

The capacity of EDTA to inhibit antibody-induced aggregation of and release from platelets treated with metabolic inhibitors indicates that this interaction does not represent agglutination, which is not inhibitable by EDTA. In contrast, even at high antibody concentrations aggregation appears partially energy dependent. With more limited concentrations of antibody both aggregation and serotonin release were substantially inhibited, and therefore are dependent on metabolic energy. Control studies indicate that the presence of metabolic inhibitors in combination with the concentrations of antibody employed in these studies did not result in platelet lysis. Thus antibody at low concentrations appears to require metabolic energy for aggregation and release, but at high concentrations relatively little metabolic energy is required for release. The fact that PGE₃ and dibutyl cAMP inhibited this partially energy-independent release reaction is further evidence that lysis did not occur at these concentrations.

Incubation of platelets with dibutyl cAMP or PGE₃ increases the level of intracellular platelet cAMP. Both of these agents, as well as the phosphodiesterase inhibitor SH869, inhibit antibody-induced aggregation (Figs. 1A, 2A, and 3A) and ¹⁴C-serotonin release (Figs. 1B, 2B, and 3B) in a dose-dependent fashion. The markedly increased concentration of these agents required to achieve equivalent inhibition of antibody-mediated aggregation and release, when compared to epinephrine or ADP, suggests a different pathway for the antibody effect. The observation that the combination of PGE₃ and SH869 enhanced inhibition of the release reaction induced by antiplatelet antibody further suggests that high concentrations of cAMP are capable of inhibiting the antibody-induced release reaction. The failure to achieve full inhibition of aggregation suggests that high platelet cAMP levels do not completely inhibit primary aggregation by antibody.

Our results suggest that antiplatelet antibody mediates the release reaction in human platelets by at least two different mechanisms. One mechanism requires metabolic energy and involves the release of ADP. This pathway is similar to that induced by exogenous ADP and epinephrine. Another pathway(s), perhaps unique for antiplatelet antibody and most evident at high antibody concentrations, is partially independent of metabolic energy, is independent of platelet ADP release, and is inhibited by high concentrations of cAMP. This reaction is independent of the prostaglandin system, but is inhibited by EDTA. Definitive proof of the existence of two pathways will require isolation and characterization of their constituents.

An increase in the cytoplasmic concentration of calcium is thought to be a critical event in platelet aggregation and release. The intracellular concentration
of platelet calcium can be increased by the use of antibiotic ionophores. The capacity of such ionophores to mediate platelet aggregation and serotonin release may be unaffected by 2-deoxyglucose or acetylsalicylic acid. Although these results are similar to our observation with antiplatelet antibody, the marked inhibition of the ionophore effect with low concentrations of PGE, and dibutyryl cAMP suggests that the antiplatelet antibody effect is not mediated primarily via an increase in platelet calcium. Calcium, however, is required, since the reaction is inhibited by EDTA and, in experiments not shown, by EGTA.

The effect of antiplatelet antibody on platelets is similar to the action of thrombin on platelets, since the effect of high concentrations of thrombin is poorly inhibitable by acetylsalicylic acid. The antibody, however, does not mediate β-glucuronidase release, and unlike thrombin its effect is only partially inhibited by antimycin A in combination with 2-deoxyglucose. Karpatkin and Siskind have observed that heterologous antiplatelet antibody and thrombin also differ in their effects on platelet glycolysis. Furthermore, the effect of the antiplatelet antibody is not altered by the addition of thrombin inhibitors.

Whether human antiplatelet antibody mediates platelet aggregation and release by similar mechanisms remains to be determined. Similarly, whether this pathway induced by heterologous antibody is operable in physiologic or pathophysiologic states in man is unknown.

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