Effects of Platelet Inhibitors on the Platelet Aggregation Induced by Plasma From Patients With Thrombotic Thrombocytopenic Purpura

By Eric C-Y Lian and N. Savaraj

Antiplatelet drugs have been used in the treatment of thrombotic thrombocytopenic purpura (TTP) but their in vivo efficacy remains controversial. It has been shown that, in vitro, the plasmas obtained from patients with TTP induced the aggregation of washed platelets from normal donors as well as patients in remission. The effects of platelet inhibitors on the TTP plasma-induced platelet aggregation were examined. It was found that aspirin, indomethacin, ibuprofen, sulfipyrazole, and esicatetraynoic acid, prostaglandin E1, prostaglandin I2, dBCAMP, apyrase, creatine phosphate/creatin phosphokinase, antimycin, 2-deoxy-D-glucose, dipyridamole, clofibrate, dextran 40, dextran 70, dibucaine, xylocaine, methylmaleimide, and ethylendiamine tetaacetic acid had little or no effect at all. These data lead us to conclude that at least in certain cases, antiplatelet drugs probably play a limited role in the treatment of patients with TTP.

**MATERIALS AND METHODS**

**TTP Plasma**

TTP plasmas were obtained from two patients with classic TTP during active disease. One of the patients, who responded to plasma infusion, was reported from this Medical Center by Drs. J. Byrnes and M. Khurana. Another patient, who did not respond to plasma infusion initially and responded to exchange blood transfusion and plasmapheresis, was described by Dr. J. Ansell et al. Approximately 450 ml of blood was collected into JF-15 Blood-Pack unit containing 63 ml of citrate phosphate dextrose (CPD) solution or Blood-Pack Unit containing 67.5 ml of anticogulant citrate dextrose (ACD) solution (Fenwall). Blood was centrifuged at 2400 g for 20 min at 4°C, after which the supernatant was decanted and spun at 10,000 g for 10 min at 4°C. The collected platelet-poor plasma was divided into small aliquots and stored at –80°C. The plasma of the second patient was a gift from Dr. J. Ansell. Both plasmas were demonstrated to possess a platelet aggregating factor, which was inhibited by normal plasma.

**Chemicals**

Aspirin, indomethacin, collagen type II, apyrase grade II, N3-2H dibutyryl cyclic 3', 5'-adenosine monophosphate (dBCAMP), ethylendiaminetetraacetic acid (EDTA), creatine phosphate, antimycin, and 2-deoxy-D-glucose were obtained from Sigma Chemical, St. Louis, Mo.; dipyridamole solution was provided by Dr. W.M. Benson, Boehringer Ingelheim, Rigefield, Conn.; sulfipyrazole and 0.25% dibucaine HCl were supplied by CIBA-GEIGY, Summit, N.J.; Clofibrate sodium salt was supplied by Imperial Chemical Industries, Macclesfield, Cheshire, England; N-ethylmaleimide, Mallincrodt Chemical, St. Louis, Mo.; 1% xylocaine, Astra Pharmaceutical Products, Worcester, Mass.; absolute pure alcohol, U.S. Industrial Chemicals; creatine phosphate/creatine phosphokinase, C.F. Boehringer and Soehne GS Mannheim, Germany; dextran 40 (10% W/V) and dextran 70 (6% W/V), Pharmacia, Piscataway, N.J.; Prostacyclin (PGI2) and prostaglandin E1 (PGE1) were gifts from Dr. John Pike, Upjohn, Kalamazoo, Mich.; 5, 8, 11, 14-ethacatetraynoic acid (ETYA) was supplied by Dr. W.E. Scott, Hoffman-LaRoche, Nutley, N.J.; Ibuprofen was obtained from Upjohn, Kalamazoo, Mich.

Indomethacin, PGE1, antimycin, and ETYA were freshly prepared in pure ethanol. Aspirin, ibuprofen, dBCAMP, PGI2, sodium salt, clofibrate sodium salt, apyrase, EDTA, sulfipyrazole, 2-deoxy-D-glucose, creatine phosphate, and creatine phosphokinase were freshly prepared and dissolved in Tris-saline buffer, pH 7.4, which contained 0.133 M NaCl, 0.015 M Tris-Cl, 0.005 M KCl, and 0.001 M MgCl2. The pH of the reagent solution was adjusted to 7.4 before being added to the platelet suspension. Collagen was prepared as described by Hoving.

**Preparation of Normal Plasma and Platelets**

Nine parts of whole blood were drawn from the antecubital vein into polyethylene tubes containing one part 3.8% sodium citrate...
using double plastic syringe technique. The platelet-poor plasma (PPP) used as a blank in the collagen-induced platelet aggregation was prepared by centrifugation at 2400 g for 20 min at 4°C. Platelet-rich plasma (PRP) was prepared by centrifugation at 180 g for 10 min at 22°C. For collagen studies, the platelet concentration of PRP was adjusted to 250 x 10^9/liter with PPP. Platelet washing was performed according to the method of Walsh et al., with slight modification. After 1/25 volume of 25% human albumin (Armour) was introduced at the bottom of conical plastic tubes, the PRP was centrifuged at 22°C for 15 min at 1650 g. The supernatant PPP was removed by siliconized Pasteur pipette. The platelets and the albumin were suspended in the same volume (as that of original PRP) of Tris-saline buffer, pH 7.4, containing 0.133 M NaCl, 0.015 M Tris-Cl, 0.005 M KCl, and 0.001 M MgCl_2. The platelets were washed twice with the same technique and then suspended in the same buffer. Platelet concentrations were adjusted to about 750 x 10^9/liter for the platelet aggregation studies.

Method to Study the Effect of Inhibitors on Platelet Aggregation

Platelet aggregation was performed in a Chrono-log platelet aggregometer using a 609 m red filter. A 0.15 ml of Tris-saline buffer solution containing proper concentration of inhibitors was added to 0.15 ml of washed platelet suspension and incubated at 37°C for 3 min, then, 0.2 ml of the mixture was transferred to the cell warmed up to 37°C in the aggregometer, which contained 0.3 ml of TTP plasma, undiluted or partially diluted with Tris-saline buffer, pH 7.4. The percentage decrease of optical density resulting from platelet aggregation was recorded. Appropriate controls without inhibitor and blanks with all reaction mixtures except platelets were always included. The concentration of inhibitors was expressed as that in the final 0.5 ml reaction mixture.

The effect of platelet inhibitors on the collagen-induced platelet aggregation was performed by addition of inhibitors to the platelet-rich plasma in the aggregometer to make a final concentration as that in the TTP plasma-induced platelet aggregation 3 min before 3 μg of collagen was added. It was shown that, at the same concentration, aspirin, ibuprofen, indomethacin, sulfinpyrazone, ETYA, PGE_1, PGI_2, dBcAMP, apyrase, and CP/CPK inhibited at least 80% of collagen-induced platelet aggregation.

RESULTS

Effects of Inhibitors of Arachidonic Acid Oxygenation

Aspirin (from 0.1 mM to 3.6 mM) and ibuprofen (0.2 mg/ml), inhibitors of cyclooxygenase, interfered with collagen-induced platelet aggregation in platelet-rich plasma but not with TTP plasma-induced platelet aggregation (Fig. 1 and Table 1). Other cyclooxygenase inhibitors, indomethacin (8 μM),32 sulfinpyrazone (0.3 mg/ml),34 and ETYA (3 μM and 60 μM)32 also had little or no effect on TTP plasma-induced platelet aggregation.

Effects of Substances That Increase Platelet cAMP

Incubation of PGE_1 (0.3-3 μM)35 or PGI_2 (60 μM)36 with platelet suspension failed to inhibit the platelet aggregation caused by TTP plasma. dBcAMP (2 mM), an analogue of cAMP, also did not affect the aggregation of platelets by TTP plasma (Table 2).

Effects of Agents That Remove ADP

Apyrase (17 U/ml), an inhibitor of aggregation due to ADP,36 had virtually no effect on the TTP plasma-induced platelet aggregation. CP (2 μM)/CPK (4 mg/ml), which removes ADP through different
### Table 1. Effects of Inhibitors of Arachidonic Acid Oxygenation

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<td>—</td>
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<td></td>
<td>3.6 mM</td>
<td>44.4</td>
<td>6</td>
<td>—</td>
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<td>None</td>
<td>47.2</td>
<td>—</td>
<td>—</td>
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<td></td>
<td>0.2 mg/ml</td>
<td>47.8</td>
<td>No</td>
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<td>39.8</td>
<td>11.1</td>
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<td></td>
<td>8 μM</td>
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<tr>
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### Table 2. Effects of Inhibitors That Increase Platelet cAMP

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<td></td>
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### Table 3. Effects of Agents That Remove ADP

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<td>Final Concentration</td>
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<td>Maximal Aggregation (%)</td>
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<td></td>
<td>17 U/ml</td>
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<td>19</td>
<td>36.7</td>
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<tr>
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<td>2 μM/4 mg/ml</td>
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### Table 4. Effects of Energy Metabolic Inhibitors

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<td>Final Concentration</td>
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<td>Maximal Aggregation (%)</td>
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<td>DOG</td>
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<td>8 mM</td>
<td>25.0</td>
<td>11</td>
<td>34.4</td>
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<tr>
<td>Antimycin/DOG</td>
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<tr>
<td></td>
<td>1.4 μM/8 mM</td>
<td>23.5</td>
<td>16</td>
<td>35.6</td>
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mechanisms,39 did not reduce the magnitude of platelet aggregation induced by TTP plasma (Table 3).

Effects of Energy Metabolic Inhibitors

Antimycin (at the concentration of 1.4 \( \mu M \)), an inhibitor of oxidative phosphorylation, did not affect the platelet aggregation induced by TTP plasma. Neither did deoxyglucose (DOG) (at the concentration of 8 mM). Preincubation with both antimycin and DOG also had little or no effect on the aggregation (Table 4).

Effects of Other Platelet Suppressants

Dipyridamole (0.04–0.08 mg/ml), an inhibitor of phosphodiesterase40 and modifier of membrane permeability to adenosine,41 did not have any effect on the TTP plasma-induced platelet aggregation. Other agents such as clofibrate (2.3 mM), dextran 40 (2%), dextran 70 (1.2%), dibucaine (0.005%), xylocaine (0.05%), methylmaleimide (50 \( \mu M \)), and EDTA (0.4 mM) virtually had little or no effect on the TTP plasma-induced platelet aggregation (Table 5).

DISCUSSION

It has been shown that the microthrombi in TTP are chiefly composed of platelet aggregates.11,42–45 The deposition of platelets in the vessels could be caused by either primary endothelial injury with secondary platelet adhesion and aggregation,45,46 or disseminated intravascular platelet aggregation as a primary event.11,43 Antiplatelet agents have been used with an attempt to disrupt or prevent the platelet aggregate formation in the therapy of TTP,1,22 but their efficacy is still questionable.33,34

The observation by us27,47 and others29,48,49 that TTP plasma causes the aggregation of autologous and homologous platelets lends support to the hypothesis that microthrombi in TTP are caused by primary intravascular platelet aggregation. This aggregation was not caused by thrombin or the coagulation process, since it was not inhibited by hirudin or diisopropylfluorophosphate.27 In order to verify the efficacy of antiplatelet drugs in the treatment of TTP, the effect of antiplatelet agents on the in vitro platelet aggregation induced by plasmas obtained from two patients with classic TTP were examined.

Nonsteroidal antiinflammatory drugs, such as aspirin, inbuprofen, indomethacin, and sulfipyrazone, inhibitors of cyclooxygenase, inhibit platelet adhesion to surface and platelet release reaction induced by surface or particulate matters (e.g., collagen and immune complexes, etc.) and secondary phase of aggregation induced by ADP and epinephrine.50 However, we found that these drugs had little or no effect on the TTP plasma-induced platelet aggregation.

PGI₂, which is synthesized by endothelial cells, prevents and reverses platelet aggregation by ADP, thrombin, and subendothelial surfaces and inhibits the release action.50 The synthesis of PGI₂ activity in the TTP patients was shown to be depressed.25,26 It was thought that infusion of PGI₂ can be used for the therapy of TTP.26 To our surprise, we found that PGI₂ failed to inhibit the platelet aggregation caused by TTP plasma. So did PGE₁ and dBcAMP, which also
stimulate the level of platelet cAMP. These observations are consistent with the recent reports that infusion of PGI$_2$ failed to raise the platelet count and save the life of two patients with TTP.$^{26,30}$

Dipyridamole, a vasodilator and inhibitor of phosphodiesterase$^{40}$ and a modifier of membrane permeability to adenosine,$^{41}$ has been reported to be beneficial in the treatment of TTP$^{13,18}$ and patients with prosthetic heart valves$^{50}$ when used in combination with aspirin. At regular therapeutic concentrations, dipyridamole does not interfere with ADP-induced platelet aggregation or release.$^{50}$ In this article we have demonstrated that dipyridamole at the therapeutic concentration had no effect on the TTP plasma-induced aggregation. The precise in vivo mechanism whereby dipyridamole inhibits thrombogenesis remains unclear.

Clofibrate was originally administered to patients with hyperlipidemia in order to lower serum lipids. This compound inhibits the second phase of ADP and epinephrine-induced aggregation.$^{50}$ However, clofibrate was found to have no effect on the TTP plasma-induced platelet aggregation.

Dextran has been used in antithrombotic therapy. Its mechanism is still not clear. In vitro, we found that neither dextran 40 nor dextran 70 affects the aggregation of platelets induced by TTP plasma.

Elimination of extracellular ADP by apyrase or CP/CPK inhibits the platelet aggregation by epinephrine and collagen.$^{38,39}$ It was found that neither apyrase nor CP/CPK inhibited TTP plasma-induced aggregation. It was shown that TTP plasma-induced platelet aggregation was not inhibited by antimiycin, DOG, or EDTA. These results indicate that TTP plasma-induced platelet aggregation is not dependent on ADP release reaction, energy generation, or extra-cellular Ca$^{2+}$.

The experimental model developed permits the in vitro examination of the effect of platelet inhibitors on TTP plasma-induced platelet aggregation. The lack of the in vitro inhibitory effect of antiinflammatory drugs and PGI$_2$ appears to coincide with the largely disappointing clinical observations. Nevertheless, antiinflammatory drugs and PGI$_2$ are capable of inhibiting the platelet adhesion$^{52,53}$ and aggregation caused by endothelial damage and of reducing the propagation of platelet aggregation caused by ADP, amine, and thromboxane A$_2$ released from aggregating platelets. Appropriate combinations and doses of various platelet suppressants may still have some ancillary therapeutic effect in vivo. A carefully designed prospective clinical study is needed in order to resolve this controversy.

ACKNOWLEDGMENT

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