Importance of Platelets in Experimental Venous Thrombosis in the Rat

By J.M. Herbert, A. Bernat, and J.P. Maffrand

Venous thrombosis was induced by ligation of the inferior vena cava in rats whose blood was made hypercoagulable by intravenous (IV) administration of tissue thromboplastin. From a dose-response showing that the administration of increasing doses of tissue thromboplastin resulted in a subsequent progressive increase of thrombus weight, two concentrations of tissue thromboplastin were chosen: a high dose (550 μL/kg, IV) where thrombus formation was optimal and a concentration (7 μL/kg, IV) where tissue thromboplastin hypercoagulability was intermediate. In both experimental conditions, leukopenia provoked by a myelotoxic drug did not influence the development of venous thrombosis. However, after thrombocytopenia induced by an antiplatelet antiserum, a dramatic decrease in thrombus formation was observed in animals that had been pre-challenged with the lower dose of tissue thromboplastin, whereas decrease in platelet count did not affect venous thrombosis under high thrombogenic challenge. When administered orally 2 hours before thrombosis induction, the ticlopidine analogue clopidogrel showed dose-dependent inhibition of thrombus formation in animals that were pre-challenged with a low dose of tissue thromboplastin (ED50 = 7.9 ± 1.5 mg/kg, orally) but remained ineffective against high tissue thromboplastin-induced venous thrombosis. We further determined the effect of heparin and hirudin, and showed that both of these drugs exhibited a more potent antithrombotic activity after injection of the lower dose of tissue thromboplastin than after injection of a high dose of tissue thromboplastin. Therefore, using our model of stasis and hypercoagulability, platelet activation played a major role in the development of venous thrombosis when the thrombogenic stimulus was mild.

© 1992 by The American Society of Hematology.

Blood COAGULATION has long been considered an important factor in the pathogenesis of venous thrombosis, a pathologic condition particularly occurring in post-traumatic and postoperative periods. Blood coagulation has long been considered pivotal in the development of venous thrombosis, but the role of platelets in this pathology is more controversial. Indeed, several investigators have suggested that platelets are not involved in the pathogenesis of venous thrombosis on the basis of experiments using antiplatelet antiserum-induced thrombocytopenia and after administration of antiplatelet agents such as aspirin. In contrast, experimental findings from other laboratories indicated that severe thrombocytopenia dramatically reduced stasis-induced thrombus formation in the rat. Furthermore, two structurally related inhibitors of platelet aggregation, ticlopidine and its analogue PCR 4099 (the racemic form of clopidogrel) exhibited a potent antithrombotic effect in this model. Both of these compounds are selective and powerful inhibitors of adenosine diphosphate (ADP)-induced platelet aggregation. They also inhibit platelet aggregation induced by low concentrations of other agonists, apparently by blocking the amplification of platelet released ADP.

The present study was undertaken to determine the relative importance of platelet activation on thrombus formation when thrombosis was induced by either high or low dosage of tissue thromboplastin. We also compared the potency of the antiplatelet agent clopidogrel with that of standard heparin and hirudin RHV2-Lys 47.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (Charles River, Saint Aubin Les Elbeuf, France) weighing 250 to 300 g were used throughout the study. New Zealand rabbits (Charles River, France) (2.5 to 3.0 kg) were used to produce antirat platelet antiserum.

Stasis-induced thrombosis after injection of tissue thromboplastin. Thrombus formation by a combination of stasis and hypercoagulability was induced as described by Vogel et al. Rats were anesthetized with sodium pentobarbital (30 mg/kg, intraperitoneally [IP]). The abdomen was surgically opened, and after careful dissection the vena cava was exposed and dissected free from surrounding tissue. Two loose sutures were prepared 0.7 cm apart on the inferior vena cava and all collateral veins were ligated. A catheter was introduced into the left carotid artery for collection of blood samples. Diluted tissue thromboplastin at the indicated doses (1 mL/kg) was injected during 30 seconds into the dorsal vein of the penis. Ten seconds after the end of the injection, stasis was established by tightening the two sutures, firstly the proximal and then the distal. The abdominal cavity was provisionally closed and stasis was maintained for 10 minutes. The cavity was then re-opened, the ligated segment was removed, opened longitudinally and the formed thrombus was removed, rinsed, blotted on filter paper, dried for 1 hour at 60°C, and weighed. Test compounds or the vehicle were administered intravenously (IV) in the dorsal vein of the penis 5 minutes or orally by gavage 2 hours before the IV injection of tissue thromboplastin. Blood samples were collected from the carotid artery into an EDTA solution (final concentration: 4.4 mmol/L) before tissue thromboplastin injection. Thrombocytopenia and leukopenia were assessed with a Baker Instrument Hematology cell counter (system 8000; Allentown, PA).

Induction of thrombocytopenia. Rats were treated with a specific antirat platelet antiserum produced in rabbits according to Ashida and Abiko. Briefly, antiplatelet antiserum was prepared by injecting rabbits with a suspension of intact rat platelets (≈1010 platelets/animal) twice a week for 2 weeks. The rabbit serum was then prepared, centrifuged (3,000g; 15 minutes), and heated at 56°C for 45 minutes. The serum was then adsorbed three times with a washed rat erythrocyte suspension and was then ready for use. A single 1.5-mL subcutaneous injection of antiplatelet antiserum induced a drop in platelet count of about 90% within 48 hours. Thrombus induction was performed 48 hours after injection of antiplatelet antiserum.

From the Haemobiology Research Department, Sanofi Recherche, Toulouse, France.

Submitted December 3, 1991; accepted July 2, 1992.

Address reprint requests to J.M. Herbert, Haemobiology Research Department, Sanofi Recherche, 195, Route d’Espagne, 31086 Toulouse Cedex, France.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1992 by The American Society of Hematology.

0006-4971/92/8009-0047$3.00/0

Induction of leukopenia. Leukopenia was obtained 48 hours after injection of mechloretamine hydrochloride as described by Lipinski and Gurewich. The drug was administered as a single IV bolus injection in the caudal vein at the dose of 1 mg/kg. This treatment did not affect platelet count but led to leukocyte counts equal to 15% to 25% of controls after 48 hours.

Drugs and dosage. Clopidogrel (d-methyl (2-chlorophenyl)-5-(4,5,6,7-tetrahydrothieno) (3,2-c) pyridinyl) acetate, hydrogensulfate) was from Sanofi Recherche (Toulouse, France). Hirudin (rHV2-Lys 47 variant) was from Sanofi Elf Bio Recherche (Labège, France). Standard heparin (162 IU/mg, sodium salt from pig intestinal mucosa) and mechloretamine hydrochloride were purchased from Sigma Chemical Co (L'Isle d'Abeau, France). All of these compounds were solubilized in saline and administered as indicated. Thromboplastin C (from rabbit brain), obtained from Baxter Dade (Dudingen, Switzerland), was reconstituted with distilled water as indicated by the manufacturer and diluted with saline before use. The tissue thromboplastin solution was kept at 0°C for the whole length of the experiment.

Statistical analysis. The results are expressed as mean ± SEM. Statistical analysis was performed by Mann-Whitney nonparametric analysis of variance taking P < .05 to indicate a significant difference.

RESULTS

Effect of tissue thromboplastin on stasis-induced venous thrombosis. We firstly evaluated the effect of increasing doses of tissue thromboplastin on thrombus formation in the venous stasis model. As shown in Fig 1, stasis alone did not cause thrombus formation within the measured period of time (10 minutes) but simultaneous administration of increasing doses of tissue thromboplastin resulted in a progressive increase of thrombus weight. After 10 minutes, a red thrombus located between the two ligatures was present in 60% to 90% of the animals injected with tissue thromboplastin in a range of doses between 2.5 and 3 μL/kg, and the incidence was 100% for doses greater than 5 μL/kg. At the highest concentration of tissue thromboplastin (550 μL/kg), the mean thrombus weight was 6.5 ± 0.3 mg (n = 20).

Fig 1. Effect of tissue thromboplastin on stasis-induced venous thrombosis in the rat. Increasing doses of tissue thromboplastin were injected IV, 10 seconds before establishment of stasis in the vena cava of the rats. Thrombus weight was measured 10 minutes later. Each point is the mean ± SEM of 10 to 20 animals.

For the following experiments, 2 doses of tissue thromboplastin were chosen: a high dose in which thrombus formation was optimal (550 μL/kg, IV) and a concentration in which tissue thromboplastin-hypercoagulability was intermediate (7 μL/kg, IV), the thrombus weight being 4.1 ± 0.2 mg (n = 20) at this dose.

Effect of thrombocytopenia and leukopenia on stasis-induced venous thrombosis. We studied the involvement of platelets on stasis-induced venous thrombosis under high and low hypercoagulability levels. The effect of thrombocytopenia, induced by an antiplatelet antiserum, on stasis-induced thrombosis is shown in Table 1. Subcutaneous injection of antiplatelet antiserum resulted within 48 hours in the reduction of platelet count to less than 10% of basal values. The thrombus weight and the incidence of thrombosis were not affected by thrombocytopenia when 550 μL/kg of tissue thromboplastin were injected before stasis induction. However, a dramatic decrease in thrombus formation was observed in animals that had been pre-challenged with a low dose of tissue thromboplastin (87% inhibition of thrombus formation).

Mechloretamine injection (1 mg/kg, IV) induced, within 48 hours, a leukocyte count reduction to about 15% to 25% of basal values (Table 2). Under these experimental conditions, the platelet count was not significantly modified by mechloretamine treatment (not shown). Table 2 reports that leukopenia did not affect stasis-induced thrombus formation after injection of both doses of tissue thromboplastin.

Effect of clopidogrel on stasis-induced thrombosis in the rat. Because platelets appear to play an important role in venous thrombosis under low thrombogenic challenge, clopidogrel, a selective anti-ADP antiplatelet agent, was tested for its ability to affect venous thrombosis. Under high thrombogenic challenge (550 μL/kg, IV), clopidogrel (25 mg/kg, orally) administered orally 2 hours before tissue thromboplastin injection, did not affect (P > .05) thrombus formation (Fig 2), whereas after injection of a low dose of tissue thromboplastin (7 μL/kg, IV), a potent dose-dependent antithrombotic effect was observed. The ED50 value (dose that inhibited 50% of thrombus formation, calculated on the basis of the linear regression of dose-response curves) was 7.9 ± 1.5 mg/kg, orally (n = 10).

When clopidogrel was administered orally (25 mg/kg, orally) 2 hours before stasis-induced thrombosis in the presence of increasing doses of tissue thromboplastin, dependence of the antithrombotic effect of clopidogrel on the intensity of the thrombogenic challenge was confirmed (Fig 3). Indeed, whereas clopidogrel displayed a significant antithrombotic effect after the administration of 40 μL/kg of tissue thromboplastin, it reached its maximum protective effect at a concentration of tissue thromboplastin between 5 and 10 μL/kg, IV.

Effect of heparin and hirudin (rHV2-Lys 47) on stasis-induced venous thrombosis. IV bolus injection of standard heparin performed 5 minutes before stasis induction dose-dependently reduced thrombus weight in both experimental conditions of stasis (Fig 4). In the most severe state of hypercoagulability (tissue thromboplastin concentra-
PLATELETS AND VENOUS THROMBOSIS

Table 1. Effect of Thrombocytopenia on Stasis-Induced Thrombosis Performed in the Presence of Tissue Thromboplastin

<table>
<thead>
<tr>
<th>Tissue Thromboplastin</th>
<th>Platelet Counts (x 10^9/mL)</th>
<th>Thrombus Weight (mg)</th>
<th>% Inhibition of Thrombosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>550 μL/kg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>890 ± 48</td>
<td>4.4 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>Thrombocytopenia</td>
<td>13 ± 3</td>
<td>4.0 ± 0.3</td>
<td>9*</td>
</tr>
<tr>
<td>7 μL/kg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>900 ± 36</td>
<td>3.7 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>Thrombocytopenia</td>
<td>11 ± 1.5</td>
<td>0.48 ± 0.1</td>
<td>87%*</td>
</tr>
</tbody>
</table>

Thrombocytopenia was induced by subcutaneous injection of a rat antplatelet antiserum (1.5 mL/kg, subcutaneously). Tissue thromboplastin was administered 48 hours later (7 or 550 μL/kg, IV) and stasis-induced thrombosis was induced in the vena cava as indicated under Materials and Methods. Results shown represent the mean ± SEM of 10 animals. Mann-Whitney U-test was used to determine statistical significance between vehicle- and drug-treated groups: *P < .05; **P < .01.

Table 2. Effect of Leukopenia on Stasis-Induced Thrombosis Performed in the Presence of Tissue Thromboplastin

<table>
<thead>
<tr>
<th>Tissue Thromboplastin</th>
<th>Leukocyte Counts (x 10^9/mL)</th>
<th>Thrombus Weight (mg)</th>
<th>% Inhibition of Thrombus Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>550 μL/kg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>8.2 ± 0.5</td>
<td>6.03 ± 0.6</td>
<td>0*</td>
</tr>
<tr>
<td>Mechlorethamine</td>
<td>2.1 ± 0.5</td>
<td>6.10 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>7 μL/kg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>9.1 ± 0.4</td>
<td>3.65 ± 0.2</td>
<td>0*</td>
</tr>
<tr>
<td>Mechlorethamine</td>
<td>1.4 ± 0.1</td>
<td>3.79 ± 0.5</td>
<td></td>
</tr>
</tbody>
</table>

Leukopenia was induced by IV injection of mechlorethamine (1 mg/kg, IV). Tissue thromboplastin was administered 48 hours later (7 or 550 μL/kg, IV) and stasis-induced thrombosis was induced in the vena cava as indicated under Materials and Methods. Results shown represent the mean ± SEM of 10 animals. Mann-Whitney U-test was used to determine statistical significance between vehicle- and drug-treated groups: *P < .05.

Fig 2. Effect of clopidogrel on stasis-induced venous thrombosis in the rat. Clopidogrel was administered orally at the indicated doses, 2 hours before the induction of stasis performed in combination with tissue thromboplastin: 550 μL/kg, IV (○) or 7 μL/kg, IV (■). Each data point is the mean of 10 animals. Mann-Whitney U-test was used to examine the difference between vehicle- and drug-treated groups: *P < .05; ***P < .001.

DISCUSSION

The high incidence of postoperative deep vein thrombosis and closely associated pulmonary embolism has prompted investigators to carefully examine the etiology of venous thrombosis. The relative contribution of stasis, altered coagulability of the blood, vessel wall damage, and circulating leukocytes or platelets to the pathogenesis of venous thrombosis remains in dispute. Nevertheless, it is now

Fig 3. Effect of increasing doses of tissue thromboplastin on the antithrombotic activity of clopidogrel. Clopidogrel was administered orally (25 mg/kg, orally) 2 hours before the stasis-induced thrombosis in the presence of the indicated doses of tissue thromboplastin. Results shown represent the mean of 10 animals. Mann-Whitney U-test was used to examine the difference between vehicle- and drug-treated groups: *P < .05; **P < .01; ***P < .001.
known that a hypercoagulable state contributes significantly to the thrombogenic process.\(^1\) Animal models based on these activation mechanisms have been designed with the aim of studying potential antithrombotic drugs. The Wessler venous stasis thrombosis model consists of inducing blood stasis in the jugular vein of rabbits after prior injection of a procoagulant substance\(^\text{15,19}\) but it is now admitted that the nature of the thrombogenic challenge influences the pathophysiologic state of the model. In agreement with other investigators,\(^\text{14,18,19}\) we found that neither short duration stasis nor hypercoagulability alone were sufficient to induce experimental thrombosis, whereas when associated with venous stasis, tissue thromboplastin increased thrombus formation in a dose-dependent manner.

To study the relative importance of leukocytes and platelets and the efficacy of anticoagulant compounds in thrombus formation, we performed all of the following experiments under two experimental conditions: (1) A high hypercoagulable state in which tissue thromboplastin was administered at a high concentration (550 \(\mu\text{L/kg, IV}\)) before stasis induction. This experimental condition corresponded roughly to that already used by numerous investigators.\(^\text{14,20}\) (2) A low dosage of tissue thromboplastin (7 \(\mu\text{L/kg, IV}\)), resulting in the formation of a smaller thrombus generated under lower hypercoagulability.

Activated platelets provide a catalytic surface for activation of blood coagulation.\(^\text{21}\) They contribute to the initiation of clotting by the development of procoagulant activities on their phospholipid membrane and their role in triggering the endothelial expression of procoagulant and proadhesive activities is now attracting increasing attention.

This study shows that experimentally induced thrombocytopenia strongly affected thrombus formation under low thrombogenic challenge, whereas it was without effect after IV administration of a high dose of tissue thromboplastin before stasis. Therefore, these observations contradict already published results\(^\text{1,3,5,6,14}\) which indicated that platelets play a negligible role in venous thrombus formation in animals. However, the method used to produce thrombocytopenia could have contributed to the hypercoagulable state.\(^3\) Therefore, our experiments were designed to obtain marked thrombocytopenia in association with a negligible effect on leukocyte count and on blood coagulation. This was obtained by treating the animals with an antiplatelet antiseraum 48 hours before stasis-induced thrombosis.

The preventive action of antiplatelet agents on venous thrombogenesis has been studied but their activity is today highly controversial.\(^\text{2,4,5,8,22,23}\) Therefore, we demonstrated that under low thrombogenic challenge, clopidogrel was potnet, ADP-selective antiplatelet agent, was able to inhibit venous thrombosis within the same range of concentrations as that already used in another thrombosis model of a more well-recognized platelet-dependent nature, ie, arteriovenous shunt, stainless steel wire coil in the rat (A.B., manuscript in preparation), or cyclic flow variations in the dog.\(^\text{24}\) The etiology of such an effect has yet to be determined but the pro-aggregating effect of low concentrations of thrombin has been demonstrated to be ADP-dependent, whereas at higher concentrations of thrombin, ADP appears to play a minor role in its aggregant activity.\(^\text{25}\) Therefore, one can postulate that, under low thrombogenic challenge, clopidogrel is able to inhibit thrombin-induced platelet aggregation in vivo, whereas when circulating thrombin concentrations are higher, clopidogrel cannot overcome thrombin-induced activation of platelets.

Because polymorphonuclear neutrophils (PMNs) were observed to be the first cells to adhere to the vascular
endothelium following vein stasis, several investigators have provided evidence that leukocytes contribute to the initiation of venous thrombosis by producing endothelial damage.\cite{26,27} Hypoxia has been proposed as a trigger for the induction of the pro-adhesive and pro-coagulant activity of both vascular endothelium and monocytes/macrophages.\cite{28} Therefore, leukocyte invasion that occurs in response to trauma to adjacent tissue followed by stasis at the site of injury may cause endothelial damage and procoagulant expression that becomes a locus for thrombus formation.\cite{28,29,30} Studies from our laboratory confirmed these observations. Indeed, a 50% decrease in white blood cell count was sufficient to strongly inhibit thrombus growth (72% inhibition, \( P < 0.01 \)) when venous thrombosis was induced by prolonged stasis (3 hours) without the administration of a thrombogenic challenge. However, after administration of tissue thromboplastin, no effect of leukopenia on thrombus formation could be observed. Most probably, the reason for this difference in the effect of leukopenia on thrombus growth is related to the duration of venous stasis (3 hours when thrombosis was induced by stasis alone v 10 minutes under hypercoagulability state). Indeed, the stimulation of leukocytes by a variety of agents to develop procoagulant activity require a longer time period to be significant.\cite{31}

The importance of activation of the clotting system in the occurrence of venous thrombosis is now evident and our data confirmed that anticoagulant compounds like heparin and hirudin rHVZLys 47, a direct thrombin inhibitor, displayed marked antithrombotic activity in stasis-induced thrombosis in the rat. To our knowledge, this is the first observation that the antithrombotic activity of anticoagulant compounds may vary depending on the degree of hypercoagulability induced by increasing amounts of tissue thromboplastin. However, other investigators have already observed that the antithrombotic effectiveness of sulphated polysaccharides may vary depending on the stimulus that initiates thrombus formation.\cite{32,33,34}

The mechanism of the antithrombotic effect of antiplatelet drugs in a venous thrombosis model is uncertain. It could either influence the procoagulant effect of platelets or the effect of platelets on endothelium. Although the molecular determinants of this effect are uncertain, the practical clinical implication is that antiplatelet agents may be effective in preventing venous thrombosis clinically.

**ACKNOWLEDGMENT**

The authors acknowledge A.J. Patachini for her helpful comments on the manuscript. We also thank A. Mur and G. Mauran for expert technical assistance.

**REFERENCES**

Importance of platelets in experimental venous thrombosis in the rat

JM Herbert, A Bernat and JP Maffrand