Assessment of the Hemostatic Effectiveness of Human Platelets Treated With Aminomethyltrimethyl Psoralen and UV A Light Using a Rabbit Ear Bleeding Time Technique

By Stephen J. Wagner, Leslie Bardossy, Gary Moroff, Roger Y. Dodd, and Morris A. Blajchman

The photochemical aminomethyltrimethyl psoralen (AMT), in conjunction with UV A light (UVA), has been shown to inactivate human immunodeficiency virus-1 and model viruses in platelet suspensions under conditions that have only a minimal effect on in vitro platelet properties. A rabbit ear bleeding time technique was used to assess the hemostatic effectiveness of human platelet suspensions treated with AMT/UVA. New Zealand White rabbits were made thrombocytopenic by a combination of irradiation and heterologous antirabbit platelet antisemur. Reticuloendothelial function in these rabbits was suppressed by the intravenous administration of ethyl palmitate. The hemostatic function of 1- and 5-day-old human platelet suspensions (14.5% plasma) that had been treated on day 1 with 40 ng/mL AMT and 24 kJ/m² UVA (1 X UVA) was evaluated by measuring microvascular bleeding times after a standard incision. Comparable bleeding times were observed after infusion with both control and AMT/UVA-treated platelets stored for either 1 or 5 days. With the transfusion of AMT/1 X UVA-treated platelets stored for 5 days, the mean (± SD) bleeding time was 156.3 ± 39.2 seconds (n = 10). With untreated platelets (no AMT/no UVA), stored for 5 days, the mean bleeding time was 189.2 ± 36.4 seconds (n = 10). Neither AMT nor 1 X UVA treatment alone influenced the observed bleeding times. In contrast, the hemostatic effectiveness of human platelet suspensions was diminished if they were exposed to three times the standard UVA dose (72 kJ/m²) on day 1 and stored for 4 more days, regardless of whether AMT was present, with the mean bleeding time increasing to 442.2 ± 122.6 seconds (n = 15, AMT present) or 396.0 ± 45.9 seconds (n = 10, AMT absent). These results are consistent with data obtained from in vitro studies and indicate that virucidal AMT/1 X UVA treatment does not influence platelet hemostatic function. However, the final conditions to achieve these results must be carefully controlled.

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Although such results were encouraging, we considered it essential to determine whether phototreated platelets maintain normal hemostatic function throughout storage. In this report, we investigated, using an animal model, the hemostatic effectiveness of AMT/UVA-treated human platelets.

MATERIALS AND METHODS

Chemicals and solutions. AMT was obtained from HRJ Associates (Concord, CA), and stock solutions were prepared in distilled water to a final concentration of 2.4 mg/mL and sterilized by filtration using a 0.22-μm filter (Millipore, Bedford, MA). A nonplasma isotonic additive storage medium (SM) solution developed to store platelets was used as a platelet resuspension medium.

Preparation of platelets. Platelet concentrate suspensions were prepared by the Canadian Red Cross Society (Hamilton, Ontario) from units of whole blood (450 ± 50 mL) drawn into citrate-phosphate-dextrose-adenoine (CPDA-1; 63 mL) using Quad Pack collection containers (Fenwal Laboratories, Deerfield, IL). Platelet-rich plasma was prepared within 4 hours of phlebotomy by centrifugation at 1,500g (Hettich Zentrifugen Roto Silenta/RP, Tuttlingen, Germany) for 4 minutes at 20°C to 24°C. Concentrated platelet suspensions were prepared by centrifugation of the platelet-rich

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plasma at 3,000g for 11 minutes, followed by expression of as much plasma as possible from the platelet pellet so that only approximately 10 mL remained, and by subsequent addition of 50 mL SM. Platelets were allowed to rest for approximately 1 hour before resuspension and storage at 20°C to 24°C with agitation. On day 1, the platelets were transferred to PL269 containers (Fenwal Laboratories) by using a sterile connecting device (SCD 312; Haemonetics, Braintree, MA). One milliliter of a 2.4 mg/mL AMT stock or 1 mL sterile distilled water (in control units) was then added by syringe through a sterile medication site (Fenwal Laboratories). The resulting platelet suspension was thoroughly mixed.

**Light source and irradiation.** UVA light was delivered by using a prototype ultraviolet irradiator (Fenwal Laboratories) containing two banks of six UVA bulbs (BLE-IT151; Spectronics Corporation, Westbury, NY). Before irradiating the various human platelet suspensions, the bulbs were turned on for at least 5 minutes to stabilize the light output. The amount of light output delivered to the surface of the test samples was determined by using a light meter (model IL1400 equipped with a detector, filter and diffuser SEL033/UVA/W; International Light, Newburyport, MA). The fluence rate, or energy flux (J/m²/s) per second was approximately 131 W/m² (W = k/s) for each light bank.

Platelet suspensions were irradiated from above and below in UV-permeable PL269 platelet storage containers. During UVA irradiation, units were agitated at 100 to 150 RPM using an orbital shaker (Lab-Line Instruments Inc, Melrose Park, IL) that was modified to include a Fenwal-supplied bracket containing a quartz stage.

All platelet suspensions were treated on day 1. Samples were treated under one of the following conditions: 40 µg/mL AMT alone; AMT with 24 kJ/m² UVA (1X); AMT with 72 kJ/m² UVA (3X); UVA alone (no AMT); ΔX UVA alone (no AMT); or were left untreated. Based on previous studies, these light fluences (energy flux [J/m²] or fluence rate x time), in conjunction with 40 µg/mL AMT, defined the boundary conditions for inactivating virus while maintaining in vitro platelet function.

**Storage of platelet suspensions.** After UVA irradiation, control and AMT/UVA-treated day-1 platelet suspensions were stored for 4 additional days in PL269 containers at 20°C to 22°C with end-over-end agitation (Platelet Agitator; Helmer Laboratories, St Paul, MN) before performing the rabbit bleeding assay.

**Rabbit ear microvascular bleeding time technique.** New Zealand White rabbits were rendered thrombocytopenic according to a previously published protocol. In brief, 2.5- to 3.0-kg animals were γ-irradiated using a 137-Cs source (930 cGy). The ears of the rabbits were shielded with a lead ribbon during irradiation to avoid vessel damage. Platelet counts began to decrease 4 days after irradiation and decreased to between 10 and 80 × 10⁹/L between days 7 and 9. Sheep antirabbit platelet serum was administered intravenously on day 8 at a dose of 0.14 mL/kg. After the intravenous infusion of the heterologous antiserum, platelet counts declined to less than 10 × 10⁹/L within 30 minutes. An ethyl palmitate suspension was also administered intravenously (1 g/kg) on day 8 to suppress the rabbit’s reticuloendothelial system. This manipulation increased the half-life of infused human platelets from less than 5 minutes to approximately 4 hours and enabled the administration of human platelets, which remained in the circulation long enough to correct the prolonged bleeding time of the thrombocytopenic rabbits.

In contrast, normal rabbit platelets have an in vivo half-life of approximately 40 hours. In this model, fresh human platelets gave a correction of the ear bleeding time comparable to that of fresh rabbit platelets at similar platelet counts.

The hemostatic function of stored human platelet concentrates was evaluated in rabbits using a previously described microvascular bleeding time technique. Before measuring the microvascular bleeding time on day 9 postirradiation, the rabbits were anesthetized by intravenous administration of 60 mg/kg of sodium pentobarbital.

The rabbits received an infusion through an ear vein of 10 mL of either a control or a phototreated platelet suspension. One hour later, the other ear was immersed in a 37°C circulating bath containing 0.9% saline for 5 minutes. An incision through the ear was made at a site that avoided visible vessels and the ear was reimpressed in the 0.9% saline bath. The time required for visible bleaching to cease was recorded. For each experimental condition, two bleeding time determinations were performed in at least 10 different rabbits receiving platelet suspensions derived from 10 different donors. The final bleeding time recorded was the mean of the two determinations. A platelet count was performed after each bleeding time determination, with the reported platelet count recorded as the mean of these two determinations. As previously described, most thrombocytopenic rabbits that received no platelet infusion had bleeding times greater than 900 seconds.

**Statistical methods.** Descriptive statistics included sample size, mean, and standard deviation. Inferential procedures included Student’s t-test and two-way analysis of variance using F statistics. P values were expressed as two-tailed. The normality of data distribution was checked with rootogram analysis, a procedure that compares a histogram of data to a Gaussian distribution. The level of significance was chosen at .05. All analyses were conducted using Minitab Version 7.2 (Minitab, Inc, State College, PA) run on a microcomputer.

### RESULTS

A comparison of the ear microvascular bleeding times obtained in thrombocytopenic rabbits that received control (no AMT/UVA treatment) or AMT/1X UVA-treated platelet suspensions stored for 1 day (no storage after phototreatment) is given in Table 1. There was no significant difference between mean bleeding times of animals receiving AMT/UVA-treated and control platelet suspensions. In the absence of any platelet transfusions, bleeding times exceeded 900 seconds (data not shown), which agreed with results from previous studies.

In such animals, the platelet counts were invariably less than 10 × 10⁹/L.

The bleeding times of thrombocytopenic rabbits that received control and phototreated platelet suspensions stored for 5 days (ie, 4 days after AMT/UVA treatment) are shown in Table 2. Results were similar to those obtained with day-1 platelets. Animals receiving control (untreated) 5-day-old platelet suspensions had a mean bleeding time of 189.2 ± 36.4 seconds, compared with 159.5 ± 62.2 seconds in animals receiving AMT/1X UVA-treated platelet suspensions stored for 5 days. These results are not significantly different from each other. Similar bleeding times were also observed.

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**Table 1. Rabbit Microvascular Ear Bleeding Times for AMT/UVA-Treated and Untreated Control Human Platelet Suspensions Stored for One Day**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Platelet Count (×10⁹/L)</th>
<th>Bleeding Time (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No AMT, no UVA</td>
<td>10</td>
<td>130.0 ± 36.4</td>
<td>158.4 ± 14.7</td>
</tr>
<tr>
<td>AMT/1X UVA</td>
<td>10</td>
<td>124.7 ± 38.0</td>
<td>155.0 ± 19.6</td>
</tr>
</tbody>
</table>

Platelet counts (mean ± SD) and bleeding times (mean ± SD) were measured 60 minutes subsequent to the infusion of the human platelet suspensions.
in animals receiving transfusions of platelet suspensions treated with AMT alone (no UVA) or 1× UVA alone (no AMT).

However, when the platelet suspensions were exposed to three times the standard dose of UVA light, a significant lengthening of rabbit microvascular ear bleeding time was observed. Animals receiving either 3× UVA alone–treated (72 J/m²) or AMT/3× UVA–treated platelet suspensions (day 5) had mean bleeding times of 396.0 ± 45.9 and 442.2 ± 122.6 seconds, respectively, compared with the 189.2 ± 36.4 second bleeding time of rabbits receiving untreated, 5-day-old platelet suspensions. These differences were statistically significant from control (P < .01 and .001, respectively).

**DISCUSSION**

The psoralens, 8-methoxypsoralen (8-MOP) and AMT,7-9,21 in conjunction with UVA light, have been used to study the feasibility of viral inactivation in platelet concentrates. For 8-MOP, conditions (drug concentration, light fluence, and oxygen tension) were identified by Lin et al5 whereby 5 log₁₀ of virus were inactivated with minimal alteration of in vitro platelet properties during storage. Dodd et al,7 working with AMT and platelets suspended in a medium consisting of approximately 10 mL plasma and 50 mL SM, described 2 sets of virus inactivation conditions (normal and low oxygen tensions), which resulted in 5 log₁₀ inactivation of model virus and maintenance of the in vitro properties of platelets during storage. In that study, the use of plasma reduced platelet suspension diminished the inhibition of virus inactivation5 caused by competitive binding of AMT to serum albumin.22

Although platelet aggregation was not influenced by AMT/UVA treatment, none of these studies has addressed whether the treated platelet suspensions retain their in vivo hemostatic function. In this report, we provide in vivo data indicating that the transfusion of AMT/UVA-treated human platelets to thrombocytopenic rabbits results in ear bleeding times that are indistinguishable from those observed in animals receiving untreated platelets. Similar results were obtained with AMT/UVA-treated platelets that were stored for 1 day or 5 days before transfusion.

Further studies are required to address the issue as to whether similar results would be obtained in humans. However, based on previous evidence, the rabbit bleeding time model appears to be predictive of results obtained in humans. For example, platelets that have been stored for 120 hours at 22°C maintain normal hemostatic function in the rabbit model, as they do in humans; whereas platelets stored at 4°C do not.20 Recently, we have also shown that platelets stored for up to 5 days in low plasma volumes (30 to 35 mL) maintain hemostatic function in vivo in the rabbit model and also are viable in vivo in humans.23

The maximum dose of UVA used in AMT/UVA treatment that is virucidal yet not toxic to platelets is similar regardless of whether it is measured by maintenance of hemostatic effectiveness or by preservation of in vitro platelet function. Based on the data provided by this study and those reported previously by Dodd et al,7 there appears to be a deterioration of both in vitro platelet function and hemostatic effectiveness when the UVA dose is increased from the standard dose of 24 kJ/m² to 72 kJ/m² (3× UVA). This effect appears to be independent of the presence of AMT (Table 2, UVA alone; Moroff et al, unpublished results) and emphasizes the importance of careful control of UVA fluence.

Considerably more information is required before it would be possible to implement a viral inactivation protocol based on AMT and UVA. This includes, in addition to clinical trials evaluating both hemostatic effectiveness and in vivo platelet survival in humans, the development of techniques to remove residual psoralens to diminish the potential for damage to the nucleic acids of cells that has been reported with some psoralens.22,24,25 Nevertheless, the demonstration that AMT/UVA-treated platelet suspensions retain hemostatic function is highly encouraging, suggesting that viral inactivation of platelet concentrates is achievable without compromising platelet function.

**ACKNOWLEDGMENT**

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**REFERENCES**

2. Petersen LR, Statten G, Dodd R: Time period from infectiousness as blood donor to development of detectable antibody and the risk of HIV transmission from transfusion of screened blood. Transfusion 32:465S, 1992 (abstr)

**Table 2. Rabbit Microvascular Ear Bleeding Times for AMT/UVA-Treated or Control Human Platelet Suspensions Stored for 5 Days**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Platelet Count (x10⁶/L)</th>
<th>Bleeding Time (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No AMT, no UVA</td>
<td>10</td>
<td>159.5 ± 62.2</td>
</tr>
<tr>
<td>No AMT, 1× UVA</td>
<td>10</td>
<td>149.6 ± 24.0</td>
</tr>
<tr>
<td>No AMT, 3× UVA</td>
<td>10</td>
<td>127.9 ± 45.5</td>
</tr>
<tr>
<td>AMT + 1× UVA</td>
<td>10</td>
<td>123.6 ± 22.2</td>
</tr>
<tr>
<td>AMT + 3× UVA</td>
<td>10</td>
<td>164.5 ± 64.2</td>
</tr>
<tr>
<td>AMT + 3× UVA</td>
<td>15</td>
<td>172.5 ± 67.7</td>
</tr>
</tbody>
</table>

*Platelet counts (mean ± SD) and bleeding times (mean ± SD) were measured 80 minutes subsequent to the infusion of human platelet suspensions.*


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