Inhibition of Platelet Adhesion to Surfaces of Extracorporeal Circuits by Disintegrins

RGD-Containing Peptides From Viper Venoms

Jacek Musial, MD, Stefan Niewiarowski, MD, PhD, Boguslaw Rucinski, MD, PhD,
Gwendolyn J. Stewart, PhD, Jacquelynn J. Cook, PhD,
Janice A. Williams, PhD, and L. Henry Edmunds Jr., MD

Previous studies indicate that exposure of fibrinogen receptors associated with glycoprotein IIb/IIIa complex contributes to platelet loss during cardiopulmonary bypass. Recently, we isolated a number of RGD (Arg-Gly-Asp)-containing, low molecular weight, cysteine-rich peptides from viper venoms. These peptides, which we propose to call “disintegrins,” block platelet-fibrinogen interaction and platelet aggregation. We compared the effect of RGDS (Arg-Gly-Asp-Ser) and four disintegrins (echistatin, flavoridin, albolabrin, and bitistatin) on platelet behavior in a membrane oxygenator. During simulated extracorporeal circulation for 2 hours, platelet count decreased to about 30% of initial values. Addition of echistatin (60–200 nM), albolabrin (60–200 nM), bitistatin (60 nM), and flavoridin (45 nM) significantly inhibited platelet loss in the circuit. RGDS (33 μM) did not show any significant inhibitory effect. ADP-induced platelet aggregation was inhibited in samples of platelet-rich plasma taken from the circuits containing disintegrins. However, echistatin appeared to be a more potent inhibitor of platelet aggregation, whereas albolabrin and flavoridin interfered more selectively with platelet loss from the circuit. Echistatin prevented the accumulation of glycoprotein IIIa on the surface of the circuit. Echistatin (60–200 nM), flavoridin (45 nM), bitistatin (60 nM), and albolabrin (200 nM) significantly inhibited the loss of β-thromboglobulin from platelets into circulating plasma. Electron microscopy studies demonstrated shape change but not degranulation in platelets circulating in the presence of 200 nM echistatin. On the other hand, this peptide (up to 1,000 nM) did not prevent loss of α granules and β-thromboglobulin from thrombin-stimulated platelets, although it prevented their aggregation. In conclusion, disintegrins protect platelets in the circuit by preventing their adhesion to surfaces and, therefore, preventing fragmentation of adhered platelets under the shear stress of flowing blood. This study indicates that disintegrins may be potential candidates for platelet protection during cardiopulmonary bypass. (Circulation 1990;82:261–273)
ANTI-CYTOADHESIVE PEPTIDES FROM SNAKE VENOMS (DISINTEGRINS)

Albolabrin:

<table>
<thead>
<tr>
<th>1</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>60</th>
<th>70</th>
</tr>
</thead>
<tbody>
<tr>
<td>EAGEDCGSPANPCCDAATCKLPGAQCGLCDDQCSFMKKGRTICRRARDDLDDYCNGISAGCPNPLHA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Flavoridin:

GEECDGSPSNPCDAATCKLPGAQCGLCDDQCRFKKTGIRCIARGDFPDRCGTLSNDCPRWNL

Echistatin:

ECSEGPCRRNCKFLKGETICKRARGDDMDYCNKTCDCPRNHPKGPAT

Bitistatin:

VSPPVCGNKILEQ-

GEDCGSPFANQCQCCNNAATCKLTPGSQCNHGECDDQCKFFKARVTICRRARDDWDDYCTGKSSDCFPNH

Figure 1. Amino acid sequences of anticytoadhesive peptides from snake venoms (disintegrins). Albolabrin was prepared from Trimeresurus albolabris, flavoridin from T. flavoviridis, echistatin from Echis carinatus, and bitistatin from Bitis arietans.

gen molecule: RGDS (Arg-Gly-Asp-Ser),<sup>13</sup> RGDF (Arg-Gly-Asp-Phe),<sup>14</sup> and tyrosyl pentadecapeptide of the C-terminal portion of the γ chain.<sup>15</sup> The concentrations of these peptides required for inhibition of platelet aggregation range from 10 to 200 μM. Recently, three RGD-containing, cysteine-rich peptides (trigramin,<sup>16,17</sup> echistatin,<sup>18</sup> and bitistatin)<sup>19</sup> were isolated from *Trimeresurus gramineus*, *Echis carinatus*, and *Bitis arietans* snake venoms, respectively. On a molar basis, trigramin (Mr, 7,500 d), echistatin (Mr, 5,400 d), and bitistatin (Mr, 9,022 d) are about 500–2,000 times more potent in blocking platelet-fibrinogen interaction and fibrinogen-dependent platelet aggregation than is RGDS. Several similar peptides showing a high degree of amino acid sequence homology with trigramin and echistatin have been isolated from other viper venoms.<sup>20–22</sup> These peptides represent a new class of proteins that we propose to call “disintegrins,”<sup>20</sup> because they interfere with the interaction of adhesive ligands with their integrin receptors. The name of each disintegrin is derived from the name of the species or subspecies of the viper. Disintegrins appear to be nontoxic, but they interfere with the formation of platelet hemostatic plugs and platelet thromboemboli formation in vivo.<sup>19,23</sup>

The purpose of this study was to evaluate the effect of RGDS and four disintegrins (echistatin, albolabrin, flavoridin, and bitistatin) on platelet behavior during simulated extracorporeal circulation.

Methods

Reagents

ADP, aprotinin, prostaglandin E<sub>1</sub> (PGE<sub>1</sub>), benzamidine, EDTA, ε-amino-n-caproic acid (EACA), soybean trypsin inhibitor (SBTI), and Triton X-100 were obtained from Sigma Chemical Co., St. Louis, Mo. Beef lung heparin was obtained from Upjohn Co., Kalamazoo, Mich. Sepharose 2B was purchased from Pharmacia, Uppsala, Sweden. Human α-thrombin was kindly provided by Dr. John W. Fenton II, Wadsworth Center for Laboratories and Research, New York State Department of Health, Albany, N.Y.

Anticytoadhesive Polypeptides

RGDS was purchased from Peninsula Laboratories, Belmont, Calif. The four disintegrins used in this study were echistatin (*Echis carinatus*), albolabrin (*Trimeresurus albolabris*), flavoridin (*Trimeresurus flavoviridis*), and bitistatin (*Bitis arietans*). The amino acid sequences of these disintegrins are shown in Figure 1. Echistatin and bitistatin were kindly provided by Dr. Paul A. Friedman, Department of Pharmacology, Merck, Sharpe & Dohme Research Laboratories, West Point, Pa. The echistatin used was obtained by chemical synthesis:<sup>24</sup> it shared identical chemical and biological properties with the peptide isolated from *Echis carinatus* snake venom.<sup>18</sup> Bitistatin was prepared by a combination of ion exchange chromatography, gel filtration, and reverse-phase high-performance liquid chromatography.<sup>19</sup> Albolabrin and flavoridin were obtained by methods developed in our laboratory by two-step reverse-phase high-performance liquid chromatography.<sup>22,25</sup> In brief, 20–40 mg lyophilized venom (*T. albolabris* venom was purchased from Latoxan, Rosans, France, and *T. flavoviridis* venom from Sigma Chemical Co., St. Louis, Mo.) were dissolved in 0.1% trifluoroacetic acid and centrifuged to remove any insoluble material. The supernatant was applied on a wide pore C-18 silica matrix column (Vydac [Separations
Group, Hesperia, Calif.)]. The gradient used was 0.1% trifluoroacetic acid containing 0–40% acetonitrile. A single active peak was identified by screening for inhibition of ADP-induced platelet aggregation. This peak was lyophilized and repurified on an identical C-18 column with a more gentle gradient. Both albolabrin and flavoridin showed only one NH2-terminal sequence. NH2-terminal sequencing of the reduced and pyridylethylated disintegrins and peptides obtained by their proteolytic degradation was performed on a gas-phase sequencer (model 120A, Applied Biosystems, Inc.) coupled to an on-line PTH analyzer (model 120A, Applied Biosystems Inc.). As seen in Figure 1, three of the disintegrins showed a high degree of homology, particularly with respect to the alignment of RGD and cysteines. Albolabrin had only eight amino acid changes from the previously characterized trigramin.17 Albolabrin, flavoridin, and echistatin are composed of 73, 71, and 49 amino acids, respectively (Figure 1). Bitistatin is composed of 83 amino acids and shows a high degree of homology with flavoridin and albolabrin. Compared with albolabrin and flavoridin, 28 amino acids are deleted from the NH2-terminal end of echistatin. On the other hand, additional amino acids are present on the NH2-terminal end of bitistatin. All four disintegrins are highly active inhibitors of ADP-induced platelet aggregation in platelet-rich plasma. The concentrations required to cause 50% inhibition of ADP-induced platelet aggregation in platelet-rich plasma (IC50) are 109 μM RGDS, 309 nM albolabrin, 105 nM bitistatin, 44 nM flavoridin, and 30 nM echistatin.

Perfusion Circuit

The experiments were performed in an in vitro model of an extracorporeal circuit that was developed and evaluated in several previous studies.1,2,11 This model provides appropriate flow geometry and hemodynamic stresses, proper surface area to blood volume ratios, and the possibility of obtaining serial measurements during a single trial.26 Each perfusion circuit had a surface area of 0.5 m2 and was assembled with standard silicone rubber tubing (Silastic, 3/8-inch internal diameter, Dow Chemical Co., Midland, Mich.), a polyvinyl chloride reservoir bag (Gish Biomedical, Santa Ana, Calif.), and a spiral coil membrane oxygenator (model 0400-2A, 0.4 m2, Sci Med Life Systems, Minneapolis, Minn.).

In control experiments, 300 ml human blood was drawn directly into a reservoir bag containing beef lung heparin (5 units/ml blood) and glucose (3.3 mg/ml blood). In addition, in each experimental study, the reservoir bag contained echistatin (20, 60, and 200 nM), albolabrin (60 and 200 nM), bitistatin (60 nM), flavoridin (15 and 45 nM), or RGDS (33 μM). Blood was drawn from healthy donors after abstinence from all medication for 2 weeks. Studies were approved by the University of Pennsylvania committee on human investigation and the National Institutes of Health.

The perfusion system was filled with blood without air formation, and the reservoir bag was immersed in a constant temperature water bath at 37°C. Blood (250 ml) was recirculated by a precisely shimmmed, barely occlusive roller pump at a rate of 0.3 l/min for 120 minutes. This rate of flow was slower than in the previous studies6,11 and thus would result in a less extensive loss of platelets from the circulation compared with previous experiments.6,11 The oxygenator was ventilated with 95% O2-5% CO2 at a rate of 0.7 l/min.

After 120 minutes, blood was drained from the entire system and replaced with two consecutive wash solutions (250 ml each): 1) 0.9% saline and 2) 0.5% Triton X-100. Both solutions contained 0.01 M EDTA, 0.3 M EACA, 500 μg/ml SBTI, and 0.001 M benzamidine. Each solution was recirculated for 15 minutes.

Sampling Procedure

Blood samples were taken directly from the donor's vein and from the reservoir bag before connection to the circuit. An initial control sample was processed immediately, and a second sample, the standing control, was processed after 120 minutes in a water bath at 37°C. Samples were obtained from the circuit at 5, 30, 60, and 120 minutes of recirculation. Additional samples of saline and Triton X-100 rinse were taken at the end of each respective 15-minute recirculation. Blood was collected after mixture with each of the following: 1) 0.11 M sodium citrate (9:1, vol/vol), 2) EDTA-PGE1 mixture (9:1, vol/vol),2 and 3) citric acid-sodium citrate–dextrose (ACD, 9:1, vol/vol). Platelet-rich plasma and platelet-poor plasma were prepared from citrated blood by differential centrifugation as described previously,11 Blood collected and mixed with ACD was centrifuged at 1,100g (20 minutes, room temperature), and the resulting plasma was recentrifuged at 13,600g (5 minutes) in a microcentrifuge. The supernatant was divided into aliquots and frozen at −70°C for further analysis. Blood taken with the EDTA-PGE1 mixture was processed similarly except that the first centrifugation was performed at 4°C.

Gel-Filtered Platelets

Gel-filtered platelets were prepared from platelet-rich plasma on Sepharose 2B columns with a modification of the procedures described by Tangen et al27 and Timmons and Hawiger.28 Columns were equilibrated with calcium-free Tyrode's buffer containing 0.1% dextrose and 0.35% bovine serum albumin. Calcium ions (10 μl of 0.1 M CaCl2) were added to 490 μl gel-filtered platelets just before the addition of the aggregating agent.

Blood Cell Counts

Whole-blood platelet counts were performed with the Unopette Microcollection System (Becton-Dickinson, Rutherford, N.J.) in a hemocytometer under phase microscopy. Platelet counts in platelet-
rich and gel-filtered plasma were performed with a Coulter Counter (Coulter Electronics, Hialeah, Fla.).

Platelet Studies

Platelet aggregation studies in platelet-rich and gel-filtered plasma were performed with an aggregometer (Chrono-Log, Havertown, Pa.). In platelet-rich plasma, threshold doses of ADP (i.e., the lowest dose of the agonist able to produce irreversible aggregation of at least 62% after 5 minutes) were determined.

β-Thromboglobulin Antigen

β-Thromboglobulin antigen was determined by means of a radioimmunoassay as previously described.29 Samples taken from the reservoir bag for β-thromboglobulin radioimmunoassay were mixed with EDTA-PGE1 solution.

Glycoprotein IIIa Antigen

Glycoprotein IIIa antigen in the Triton X-100 eluates from the perfusion circuit was identified by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis30 followed by Western blotting31 with the methodology and reagents described previously.11

Electron Microscopy

Platelet-rich plasma from samples taken from the reservoir bag (with and without echistatin) after 120 minutes of recirculation were examined. Also, gel-filtered platelets treated with various ratios of thrombin and echistatin and stirred in aggregometer tubes for 3 minutes were examined. These samples were fixed with glutaraldehyde in Tyrode’s solution containing 2% sucrose. Platelets were further fixed in 1% osmium tetroxide, stained with uranyl acetate, dehydrated with a graded ethanol series, and embedded in plastic for sectioning and further staining (uranyl acetate and lead citrate).

Statistical Analysis

The effect of various peptides on the number of platelets remaining in the circulation (% of control) and β-thromboglobulin levels at various times during recirculation were compared statistically with a two-way analysis of variance (ANOVA) (peptide by time). Because the ANOVA F tests demonstrated significant differences (acceptable α level, 0.05), Tukey’s honestly significant difference posthoc analysis was performed to determine differences between simple main-effect means. The ratios representing inhibition of platelet loss to inhibition of platelet aggregation for echistatin (60 nM), bitistatin (60 nM), flavoradin (45 nM), and albolabrin (200 nM) were compared with a single factor analysis of variance.

Results

Prevention of Platelet Loss in the Simulated Extracorporeal Circuit by Disintegrins

Figure 2 summarizes the effects of recirculation on platelet number with and without RGDS and disintegrins. The statistical analysis of the curves compared by ANOVA is presented in Table 1. Without protective agents, the platelet count in circulating blood decreased significantly. The greatest decrease, to about 30% of initial values, occurred within 5–30 minutes. By 2 hours, the platelet count had increased but did not exceed 45% of initial values. RGDS, at a concentration of 33,000 nM, appeared to have a slight protective effect that was not statistically significant. The protective effects of echistatin (60 nM), flavoradin (45 nM), and bitistatin (60 nM) were similar, whereas that of albolabrin (60 nM) was slightly less effective. Samples containing all four disintegrins were statistically different from control samples (Table 2); samples containing flavoradin were also significantly different from albolabrin samples. In addition, we observed that the increase in concentration of echistatin from 60 to 200 nM resulted in the increase of mean platelet preservation from 85.1% to 96.8%, and the increase in concentration of albolabrin from 60 to 200 nM resulted in the increase of mean platelet preservation from 66.5% to 84.2% (data not shown).

To determine the correlation between the protective effect of disintegrins on circulating platelets and their inhibitory effect on ADP-induced aggregation, we tested platelet aggregation in response to ADP in platelet-rich plasma prepared from blood samples taken from the reservoir bag before recirculation. The inhibitory effect of disintegrins on platelet aggregation was expressed in percent inhibition compared with control samples. All aggregation was performed in platelet-rich plasma stimulated at identical (threshold) concentrations of ADP. Preservation of platelets was expressed as mean platelet count in samples taken from the reservoir bag at 5, 30, 60, and 120 minutes of recirculation; platelet count before recirculation was accepted as 100%. Table 1 shows the comparison of the effect of four disintegrins on preservation of circulating platelets and inhibition of platelet aggregation in platelet-rich plasma. The ratio of the percentage of preserved platelets and inhibition of platelet aggregation was calculated for each disintegrin. When the ratio is higher, the effect on platelet aggregation is smaller, and the effect on platelet preservation is greater. Table 1 shows that the selected concentrations of the four disintegrins in this comparative study (60 nM echistatin, 60 nM bitistatin, 45 nM flavoradin, and 200 nM albolabrin) provided similar protection of platelets in the circuit (80.2–89.2%). However, echistatin had a much stronger inhibitory effect on platelet aggregation than albolabrin and flavoradin. ANOVA (Table 1) confirmed that the dissociation of the effects on platelet aggregation and platelet preservation observed in this experiment was statistically significant.

Scanning electron microscopy was used to examine the appearance of platelets adhering to the circuit surface membrane. Amorphous materials, but no intact platelets, were found on the surface of the extracorporeal circuit. This material probably represented fragmented or degranulated platelets. Accumulation of
glycoprotein IIIa antigen on the surface of the perfusion system, in extracts solubilized by Triton X-100, is shown in Figure 3 by means of SDS–polyacrylamide gel electrophoresis followed by Western blotting. In the absence of protective agents, the platelet count decreased from 224 to \(71 \times 10^3/\mu l\) during recirculation, and a high concentration of glycoprotein IIIa was present in the Triton X-100 extract (Figure 3, lanes A–E). Blood recirculated in the presence of 200 nM echistatin showed a transient drop in platelet count from 202 to \(184 \times 10^3/\mu l\). The Triton X-100 eluate from this circuit did not show detectable glycoprotein IIIa antigen (Figure 3, lane F). However, a low concentration of glycoprotein IIIa antigen was detected in the Triton X-100 eluate from a circuit exposed to recirculated blood containing 60 nM echistatin (Figure 3, lanes G and H). In this experiment, the platelet count decreased from 296 to \(253 \times 10^3/\mu l\).

**Effect of Disintegrins on Platelet Activation During Simulated Extracorporeal Circulation and on Thrombin-Induced Activation**

The effects of RGDS and disintegrins on the release of \(\beta\)-thromboglobulin antigen from platelets...
TABLE 2. Comparison of Effect of Various Peptides on Platelet Count at 5 and 120 Minutes of Extracorporeal Recirculation

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>RGDS</th>
<th>Albolabrin</th>
<th>Echistatin</th>
<th>Bitistatin</th>
<th>Flavoridin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td>−</td>
<td>−</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>RGDS</td>
<td>−</td>
<td>−</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Albolabrin</td>
<td>†</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Echistatin</td>
<td>*</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Bitistatin</td>
<td>*</td>
<td>†</td>
<td>†</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Flavoridin</td>
<td>*</td>
<td>†</td>
<td>†</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

RGDS, Arg-Gly-Asp-Ser.
All concentrations of disintegrins were 60 nM except for flavoridin (45 nM). 5 minutes, below line; 120 minutes, above line.
*p<0.01; †p<0.05 (Tukey's honestly significant difference test).

during simulated extracorporeal circulation was also examined (Figure 4 and Table 3). RGDS (30,000 nM) and albolabrin (60 nM) had no significant effect. However, echistatin (60 nM), albolabrin (200 nM), flavoridin (45 nM), and bitistatin (60 nM) blocked the release of β-thromboglobulin from platelets. Although flavoridin (45 nM) appeared to be the most effective, a higher concentration of echistatin (200 nM) had similar effects (data not shown).

The ultrastructures of platelets circulated in the presence and absence of 200 nM echistatin were compared. Echistatin does not appear to prevent platelet shape change and pseudopod projection. Platelet α granules were preserved in samples with and without echistatin (Figure 5, panels A and B).

In an attempt to explain the protective effects of disintegrins on platelets during extracorporeal circulation, we studied the effects of echistatin on

![Figure 3](http://circ.ahajournals.org/DownloadedFrom/circ.ahajournals.org)

**Figure 3. Identification of glycoprotein IIIa (GPIIIa) antigen in Triton X-100 eluates of the perfusion circuit.** Aliquots of 100 µl of the eluates were applied on sodium dodecyl sulfate-polyacrylamide gels (with 7.5% gels), and electrophoretic separation was performed according to Laemmli.24 Samples were transferred to nitrocellulose papers and stained with an antibody to the 66-kd component derived from GPIIIa as described previously.11 Molecular weight standards are indicated by the arrows. Lanes A–E: Eluate from circuits recirculated without a disintegrin (lane A, undiluted sample; lane B, 1:5; lane C, 1:10; lane D, 1:15; and lane E, 1:20). In this experiment, platelet count decreased during recirculation from 224 to 71×10^3/µl. Lane F: Eluate from circuits recirculated with 200 nM echistatin (undiluted sample). Platelet count decreased during recirculation from 202 to 184×10^3/µl. Lanes G–H: Eluate from circuits recirculated with 60 nM echistatin (lane G, undiluted sample; lane H, sample diluted 1:5). Platelet count decreased during recirculation from 296 to 253×10^3/µl.
thrombin-induced responses. In Figure 6, panels A and B show transmission electron photomicrographs of gel-filtered platelets stirred for 3 minutes in an aggregometer tube. Panel A shows that platelets stirred only with calcium chloride (control) had developed pseudopods, but α granules were present, and microtubules were located peripherally, indicating limited activation. Panel B shows platelets that were preincubated for 10 minutes with 10 μg echistatin, then stirred for 3 minutes with 1 unit/ml thrombin with the echistatin still present. Platelets rounded up and secreted essentially 100% of their α granules. Large vacuoles filled with amorphous or finely fibrillar material were always present. In about half of the cross sections, there were dense, round structures adjacent to crescent-shaped masses of material with fine and coarse fibrils. The response of platelets exposed to all proportions of echistatin and thrombin was the same as that shown in panel C. In the absence of echistatin, an aliquot of the same platelets showed extensive rounding, pseudopod formation and secretion. Aggregation was so extensive that quantitation of shape change of individual platelets was not possible. Echistatin alone had no effect on platelet shape or ultrastructure (data not shown).

Table 3. Ability of RGDS and Disintegrins to Prevent β-Thromboglobulin Release From Platelets in Extracorporeal Circuits

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Recirculation time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30 min</td>
</tr>
<tr>
<td>RGDS</td>
<td>*</td>
</tr>
<tr>
<td>Albolabrin</td>
<td></td>
</tr>
<tr>
<td>60 nM</td>
<td></td>
</tr>
<tr>
<td>200 nM</td>
<td></td>
</tr>
<tr>
<td>Bitistatin</td>
<td></td>
</tr>
<tr>
<td>60 nM</td>
<td></td>
</tr>
<tr>
<td>Echistatin</td>
<td></td>
</tr>
<tr>
<td>60 nM</td>
<td></td>
</tr>
<tr>
<td>Flavoridin</td>
<td></td>
</tr>
<tr>
<td>45 nm</td>
<td></td>
</tr>
</tbody>
</table>

RGDS, Arg-Gly-Asp-Ser.
*p<0.01; †p<0.05. Significant increase in β-thromboglobulin release compared with samples taken from circuit before recirculation. (Lack of significant increase represents protection from β-thromboglobulin release.)
Platelet images from the article:

**A** Platelets recirculated in the absence of ecdysone (panel A) and in the presence of 200 mM ecdysone (panel B).

**B** Platelets recirculated in the presence of ecdysone were similar in appearance to those recirculated in the absence of ecdysone. In both cases, platelets that remained in the plasma after recirculation had undergone modest shape change but had retained their granules.
As shown in panel D, control platelets showed a characteristic increase in light transmission; however, in the presence of echistatin, there was a limited increase in light transmission, but no large aggregates were formed as demonstrated by eye and by phase contrast microscopy. Groups of two or three platelets formed early during the light transmission increase, but their size and number did not continue to increase as light transmission increased. Release of \( \beta \)-thromboglobulin antigen was close to 100% in the presence and in the absence of echistatin (data not shown).

**Discussion**

This study shows that the disintegrins echistatin, flavoridin, bitistatin, and albolabrin, which were prepared from snake venoms, prevent platelet adhesion to surfaces of simulated extracorporeal circuits. The activity of these peptides in this experimental system exhibited limited correlation with the ability of these peptides to inhibit platelet aggregation (Table 1). That is, flavoridin and albolabrin appeared to be more selective inhibitors of platelet adhesion, whereas echistatin and bitistatin were more selective inhibitors of platelet aggregation. Because disintegrins block fibrinogen interaction with platelet receptors,16–21,25 the present data support the previously suggested contention that the interaction of the platelet glycoprotein IIb/IIIa complex with surface adsorbed fibrinogen is a major factor for platelet adhesion to these surfaces.11

Disintegrins also blocked the loss of \( \beta \)-thromboglobulin from platelets into circulating plasma (Figure 4). Our data suggest that the release of platelet constituents into the circulation takes place on the surfaces of the circuit after platelets adhere. The most plausible explanation is that the shear stress of flowing blood contributes to the loss of platelet constituents. This conclusion is supported by the observation that the majority of the circulating platelets are not degranulated (Figure 5) despite a significant increase of plasma \( \beta \)-thromboglobulin. The results of this study and an earlier one from our laboratory11 indicate that platelets that adhere undergo fragmentation. That is, although morphologically recognizable platelets are absent on the tubing, the glycoprotein IIIa antigen is present in detergent washes of the perfusion circuit (Figure 3). Platelet fragmentation during cardiopulmonary bypass also has been observed by other researchers.4,5 Flavoridin appeared to be the most potent inhibitor of the release of \( \beta \)-thromboglobulin from platelets into the plasma.

Huang et al16 reported that trigramin inhibits platelet aggregation but that it does not inhibit release of serotonin induced by thrombin or a prostaglandin \( \mathrm{H}_2 \)/thromboxane \( \mathrm{A}_2 \) analogue. Accordingly, we found that echistatin, an inhibitor of platelet aggregation induced by thrombin, did not block shape change and loss of \( \beta \)-thromboglobulin and \( \alpha \) granules from platelets stimulated by this enzyme (Figure 6). We believe that disintegrins do not prevent the platelet release reaction that follows stimulation of specific surface receptors but that they do prevent platelet adhesion and, secondarily, the fragmentation that may occur under the shear stress of flowing blood.

Levy-Toledano et al32 reported that in response to the calcium ionophore A23187, thrombasthenic and EDTA-treated platelets undergo a change in light transmission accompanied by normal release of serotonin in the absence of aggregation. This ultrastructural change (vacuolization) was responsible, at least in part, for the increase of light transmission in the absence of platelet aggregates. The platelet ultrastructural changes reported by Levy-Toledano et al32 were similar to the changes in platelets induced by thrombin in the presence of echistatin and described in our present study (Figure 6). Therefore, in our experiments, the platelet vacuolization in the presence of thrombin and echistatin could contribute to the increase of observed light transmission.

Of special interest, albolabrin and flavoridin, at concentrations that partially blocked ADP-induced platelet aggregation, provided relatively better protection of platelets in the circuit than did echistatin, which was a more potent inhibitor of platelet aggregation. This suggests that platelet adhesion to the membranes of the circuit and platelet adhesion to each other occur by different mechanisms. As mentioned previously, albolabrin and flavoridin, which are structurally more related to each other than to echistatin, appear to be more selective inhibitors of platelet adhesion. Previous studies show that trigramin (which is similar to albolabrin and flavoridin in molecular weight and sequence) binds with a much lower affinity to resting than to activated platelets.16 On the other hand, the binding affinity of echistatin to platelets is only slightly influenced by platelet activation. At present, it is difficult to explain these differences. However, one possible explanation may be the varying affinities of short compared with medium disintegrins for resting compared with activated platelets. Several relevant points have previously been established by the comparison of trigramin (medium) and echistatin (short). First, short disintegrins have a higher affinity for resting platelets than do medium disintegrins.16,18 Conversely, medium disintegrins have a higher affinity for activated platelets than do short disintegrins.16,18 Perhaps echistatin (short) is more an inhibitor of platelet aggregation in the present study, because it has already attached to the resting platelets during recirculation and is, therefore, available to prevent aggregation immediately upon stimulation. On the other hand, medium disintegrins (flavoridin and albolabrin in these experiments) would not be as likely to interact with platelets before stimulation and would, therefore, appear less potent in inhibiting aggregation. However, there would be more of these medium disintegrins available to prevent platelet adhesion to the surfaces of the circuit, because they are less likely to be bound to the circulating resting platelets. Also, the recently described platelet fibronectin receptors (glycoprotein...
Ic/Ila heterodimer)\textsuperscript{33,34} and vitronectin receptors\textsuperscript{35} may conceivably be involved in platelet adhesion to the tubing, and albolabrin and flavoridin may have higher affinity for these receptors than does echistatin.

Disintegrins appear to have a transient inhibitory effect on platelet function in vivo and a short half-life in the circulation. Cook et al\textsuperscript{23} demonstrated that infusion of trigramin into hamsters results in prolongation of bleeding time, which was normalized immediately after cessation of the infusion. In addition, radiolabeled trigramin has a short half-life in the hamster circulation (fast component, 0.7–2.0 minutes; slow component, 31–106 minutes). Similarly, infusion of bitistatin in dogs causes a significant inhibition of platelet aggregation that is fully reversible within a short period.\textsuperscript{19} Therefore, disintegrins would be effective in preventing platelet loss and adhesion during cardiopulmonary bypass and would be eliminated from the circulation shortly after the termination of the bypass.

Several previous strategies to preserve platelet numbers and function during cardiopulmonary bypass have proved disappointing or inconclusive. For example, precoating the circuit with albumin preserves platelets in the simulated extracorporeal circuit\textsuperscript{11,36} but not in vivo. Attempts to block the platelet binding sites of surface adsorbed fibrinogen\textsuperscript{12,37} have not been extended to clinical trials. The use of desmopressin acetate to increase von Willebrand factor during cardiopulmonary bypass reduced postoperative blood loss in one study\textsuperscript{10}; however, infusions of desmopressin seem to carry the risk of thrombosis, particularly in elderly patients and those with atherosclerosis.\textsuperscript{38} The vasodilatory effects of PGE\textsubscript{1} and prostacyclin (PGL\textsubscript{2}) preclude their use as platelet inhibitors during open heart surgery.\textsuperscript{39,40}

Iloprost, an experimental drug, is an analogue of prostacyclin and partially inhibits platelets by increasing platelet cyclic AMP.\textsuperscript{41} It partially protected platelets during simulated extracorporeal circulation,\textsuperscript{41} during experimental cardiopulmonary bypass in dogs\textsuperscript{42} and in patients during open heart surgery who had heparin-induced thrombocytopenia.\textsuperscript{43} These results support the theory that an antiplatelet agent would be beneficial during surgery requiring cardiopulmonary bypass. However, because iloprost administration results in dangerous hypotension,\textsuperscript{43} perhaps disintegrins can serve as an appropriate alternate for prostanooids in these circumstances. Also, the use of disintegrins and PGE\textsubscript{1} or PGL\textsubscript{2} together may be effective, because each inhibits platelets by different mechanisms. This combination therapy may adequately reduce the adverse effects of vasodilatory prostaglandins, because the amount required would be decreased.

In conclusion, we demonstrated the protective effects on platelets in simulated extracorporeal circulation of four disintegrins: echistatin, bitistatin, flavoridin, and albolabrin. Flavoridin and albolabrin appeared to be more selective inhibitors of platelet adhesion to surfaces than of platelet aggregation. Therefore, these substances may be more attractive clinically than is echistatin, because inhibition of platelet aggregation may involve risk of bleeding. At present, the side effects of the disintegrins are not known in detail, but our data indicate that these disintegrins can be infused safely in animals without detrimental effects on blood pressure or core temperature.\textsuperscript{19,21} This new class of peptides does raise the prospect of controlling the excessive blood losses associated with contemporary open heart surgery.

Acknowledgments
We acknowledge the excellent technical assistance of Lilly Schaffer, Annette Eckardt, Raymond Davis, and Weiqi-Lu. We also thank Cathy Spiotta for her secretarial assistance.
References


Key Words • glycoprotein IIb/IIIa • platelet aggregation • cardiopulmonary bypass • membrane oxygenator • viper venom • echistatin • albolabrin • bitistatin • flavoridin
Inhibition of platelet adhesion to surfaces of extracorporeal circuits by disintegrins. RGD-containing peptides from viper venoms.
J Musial, S Niewiarowski, B Rucinski, G J Stewart, J J Cook, J A Williams and L H Edmunds, Jr

_Circulation_. 1990;82:261-273
doi: 10.1161/01.CIR.82.1.261

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1990 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/82/1/261

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org//subscriptions/