Effect of Albumin on the Inhibition of Platelet Aggregation by \(\beta\)-Lactam Antibiotics

By E.M. Sloand, H.G. Klein, K.B. Pastakia, P. Pierce, and K.N. Prodouz

Platelet aggregation and bleeding time abnormalities are reported in patients receiving \(\beta\)-lactam antibiotics (\(\beta\)LAs), although clinical bleeding most frequently occurs in chronically ill, malnourished patients. Although most \(\beta\)LAs bind to serum albumin, the relative influence of bound versus unbound \(\beta\)LAs on platelet function is unknown. We examined the effect of \(\beta\)LAs on the aggregation of gel-filtered platelets from normal subjects and on platelet-rich plasma (PRP) from hypoalbuminemic patients. Therapeutic concentrations of five \(\beta\)LAs were added to normal platelets at different albumin concentrations (1.5 to 4.5 g/dL). Inhibition of aggregation by the \(\beta\)LAs was inversely proportional to the albumin concentration, and most antibiotic-treated samples showed more than 50% inhibition at albumin levels below 2.0 g/dL. When PRPs from hypoalbuminemic patients were incubated with cephalothin, aggregation was completely inhibited, in contrast to samples from patients with normal albumin levels, and this decreased platelet aggregation was partially restored (25% to 75%) by increasing the albumin concentration above 4.0 g/dL. Specific binding of \(^{14}\text{C}\)-benzylpenicillin to normal platelets decreased proportionately as the albumin concentration increased in the range of 1.0 to 5.0 g/dL. The inhibitory effects of \(\beta\)LAs on platelets in vitro appear to be influenced by albumin concentration. Plasma albumin concentration may influence bleeding in patients receiving \(\beta\)LAs.

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\(\beta\)-LACTAM ANTIBIOTICS (\(\beta\)LAs) may contribute to bleeding episodes in patients by depleting vitamin K-dependent coagulation factors,\(^3,12\) interfering with fibrin polymerization,\(^1\) or causing defects in platelet function.\(^3,7\) Despite well-documented platelet abnormalities in both normal and patient groups receiving \(\beta\)LAs,\(^8,4\) clinical bleeding primarily occurs in chronically ill and/or malnourished patients. Although platelet defects have been described at therapeutic concentrations for many antibiotics,\(^6,11\) some antibiotics produce defective platelet function in vitro only at concentrations greatly in excess of those used therapeutically.\(^3,7,12\)

\(\beta\)LAs reportedly inhibit platelet function by binding to the platelet surface, thereby preventing interaction of platelet agonists with their receptors.\(^1,11,18\) Bleeding complications in hypoalbuminemic patients receiving \(\beta\)LAs are often associated with abnormal aggregation activity in vitro, suggesting that \(\beta\)LAs may exert an effect on platelet glycoprotein receptors.\(^2\) Although most \(\beta\)LAs bind to albumin as well as to other plasma proteins,\(^18\) the effect of albumin concentration on the \(\beta\)LA inhibition of platelet function has received little attention. We investigated the \(\beta\)LA effect on platelet aggregation in the presence of varying albumin concentrations in an effort to explain the reports of bleeding in hypoalbuminemic patients receiving \(\beta\)LAs. We selected cephalothin, moxalactam, ceftriaxone, cefamandole, and penicillin to investigate \(\beta\)LA-mediated inhibition of platelet aggregation in platelets collected from patients with normal albumin levels and from hypoalbuminemic patients. In addition, we used \(^{14}\text{C}\)-benzylpenicillin to study \(\beta\)LA binding to platelets and the effect of albumin on this binding.

METHODS AND MATERIALS

Platelet sources. Platelets were obtained from three groups of individuals. Group 1 consisted of platelet concentrates from normal blood donors obtained by automated platelepheresis (Fenwal CS-3000; Baxter Health Care, Deerfield, IL). Group 2 consisted of platelet-rich plasma (PRP) samples from selected oncology patients, six with normal serum albumin (range, 3.0 to 4.6 g/dL) and four with low serum albumin (range, 2.5 to 2.8 g/dL; mean, 2.6 g/dL), studied to determine the effect of overnight incubation of platelets with cephalothin (200 \(\mu\)g/mL; Eli Lilly, Indianapolis, IN). In group 3, PRPs from patients with low levels of albumin (range, 2.6 to 3.0 g/dL; mean, 2.8 g/dL) receiving \(\beta\)LA were studied to determine the effects of adding human serum albumin in vitro. Two patients with uremia and one with cirrhosis who were receiving cephalothin (1 g six times daily) and one uremic patient receiving 250 mg of cephalothin orally four times daily were compared with one cirrhotic and six uremic patients not receiving \(\beta\)LAs. All patients and normal donors denied ingestion of aspirin, other nonsteroidal anti-inflammatory agents, or other platelet-active drugs for at least 2 weeks before this study. The hematocrits and platelet counts were similar in patients used for comparison (mean hematocrit of 22.5 in hypoalbuminemic patients and a mean of 20.0 in patients with normal albumin). Venous blood was drawn with minimal negative pressure from the antecubital vein of the arm containing an arteriovenous fistula. Blood was drawn using the double plastic syringe technique and was immediately transferred into siliconized glass tubes at room temperature, containing sodium citrate at 3.8% (vol/vol) final concentration. PRP was prepared by centrifuging the citrated blood at 1600 \(\times\) g for 15 minutes at room temperature.

Measurement of the aggregation response of normal platelets treated with \(\beta\)LAs. Platelet concentrates obtained from normal individuals were gel-filtered on Sepharose 2B (Pharmacia Fine Chemicals, Piscataway, NJ), as previously described,\(^7\) and eluted with 3.8 mM/L HEPES buffer pH 7.35 (Sigma Chemical Co, St Louis, MO), containing NaCl 0.14 mol/L, KCl 2.7 mmol/L, dextrose 1.0 g/L, NaHPO\(_4\), 3.8 mmol/L. Samples were adjusted to a platelet count of 2 \(\times\) \(10^\text{11}/\text{L}\). (Coulter Counter, Model S; Coulter Electronics, Hialeah, FL) and fibrinogen was added (final concentration, 1.5 mg/mL). The albumin level in the gel-filtered platelets, measured as previously described,\(^8\) was less than 0.4 g/dL. Sam-

From the National Heart, Lung, and Blood Institute, and the Department of Transfusion Medicine, National Institutes of Health; the Warren G. Magnuson Clinical Center, the Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD; and the Georgetown University Medical Center, Washington, DC.


Address reprint requests to E.M. Sloand, MD, NIH Building 31, Room 5421, 9000 Rockville Pike, Bethesda, MD 20892.

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samples were divided into paired aliquots and albumin levels were adjusted to different levels (1.5 to 4.5 g/dL) by the addition of fatty acid-free human albumin (25 g/dL; Sigma) or albumin for human use (Armour Pharmaceutical, Bluebell, PA). BLAs were added to one of the paired aliquots (at each albumin level) at concentrations equivalent to plasma levels achieved therapeutically: cephalothin, 100 μg/mL; moxalactam, 400 μg/mL; cefamandole, 13 μg/mL (Eli Lilly); ceftriaxone, 250 μg/mL (Roche Pharmaceutical, Nutley, NJ); and penicillin G 10 μg/mL (Squibb and Sons, Inc, Princeton, NJ). The interval between gel filtration and addition of BLA never exceeded 15 minutes.

Platelet function was measured by platelet aggregation 15 minutes after BLA addition. Each sample (0.45 mL) was incubated at 37°C for 10 minutes and aggregation was induced by adding 50 μL of 80 μmol/L adenosine diphosphate (ADP; Sigma) and stirring. The maximum slope of aggregation was determined for duplicate samples and the results were averaged. Inhibition for each albumin level was calculated as the percent of maximum slope of aggregation of the control sample at the same albumin level, but without BLA.

Aggregation response of platelets from hypoalbuminemic patients. Thirty milliliters of PRP from nine oncology patients (six with normal albumin and three hypoalbuminemic) was prepared from venous blood collected into a solution of acid-citrate dextrose (15% [vol/vol]) and centrifuged at 160g for 15 minutes at room temperature. Platelet counts ranged from 1.4 to 2.0 × 10^10/mL. Cephalothin (in phosphate-buffered saline [PBS], pH 7.4) was added to a sample of PRP at a concentration of 200 μg/mL and an equal volume of PBS was added to a control sample. The BLA-treated and untreated samples were stored overnight in modified PL 732 platelet storage bags (Fenwal; Baxter Health Care) at 22°C with constant agitation. Platelet aggregation was measured as described above. The maximum slope of aggregation in response to 8 μmol/L ADP was calculated for samples tested in duplicate.

In separate experiments, PRP from 10 hypoalbuminemic patients (four of whom were receiving cefalothin) was divided into two aliquots. Fatty acid-free human albumin (25 g/dL in 0.9% NaCl; Sigma) was added to one aliquot to adjust the albumin concentration to approximately 4.0 g/dL. An equal volume of 0.9% NaCl was added to the control aliquots. Platelet aggregation in response to 0.5 μmol/L ADP was measured as described above.

Penicillin binding experiments. A 15-μL suspension of Apiezon oil A (Biddle Co, Bluebell, PA) and n-butyl phthalate (1:9 vol/vol) together with 4 μL of [3H]-benzylpenicillin (0.28 to 4.0 nmol/mL, 27 to 162 pmol/10^9 platelets; Du Pont Co, Wilmington, DE) in ethanol (final concentration, <0.5%) was added to 200 μL samples of gel-filtered albumin-free platelets obtained from normal individuals. Samples were inverted several times, incubated at 37°C for 15 minutes, and then centrifuged at 1,200g for 15 minutes. The supernatant was removed and the tip of the tube containing the pellet was excised. The pellet was solubilized in 500 μL of 1% sodium dodecyl sulfate. Radioactivity was measured after addition of Aquasol (Du Pont Co). Binding specificity was assessed by measuring the displacement of [3H]-benzylpenicillin by 1,000-fold excess nonradioactive penicillin. Binding experiments were also performed on three gel-filtered plateletpheresis concentrates at different albumin levels. All samples were tested in quadruplicate.

**RESULTS**

Aggregation response of normal platelets treated with BLA. Aggregation was decreased significantly in gel-filtered platelets treated with any of the five BLAs tested at albumin concentrations less than 2.5 g/dL in the medium. An example of the relationship of platelet inhibition to albumin level is seen in Fig 1 when 8 μmol ADP was used as an agonist. The mean inhibition of aggregation for cephalothin in the five different platelet samples was 64% ± 35% for an albumin of 2 g/dL and 0% for an albumin of 4.0 g/dL. For most BLAs tested, platelet aggregation was either slightly affected or similar to the control sample (at the same albumin concentration but without BLA) when albumin levels exceeded 3.0 to 3.5 g/dL. Below this level, the inhibitory effect of BLAs was inversely related to albumin level in all samples. Similar effects were observed in washed platelets when 8 μmol/L ADP or thrombin (0.1 to 1.0 U/mL) were used as agonists and in gel filtered platelets when collagen and epinephrine were used (Table 1). When

![Fig 1. The albumin concentrations of gel-filtered platelets obtained from a normal donor were adjusted to levels in the range of 1.5 to 4.5 g/dL by the addition of 25 g/dL human albumin; PBS was added, where appropriate, to maintain a constant volume. The aggregation responses to 8 μmol/L ADP were determined on duplicate samples for each different albumin level before and 15 minutes after the addition of five different BLAs: (C—O) cephalothin, 100 μg/mL; (C—△) moxalactam, 400 μg/mL; (C—□) ceftriaxone, 250 μg/mL; (C—□) cefamandole, 13 μg/mL; (C—□) penicillin G, 10 μg/mL for each albumin level. These responses were compared with those of platelets at similar albumin levels but without BLA. Inhibition was calculated as the percent of the maximum slope of aggregation of the control sample at the same albumin level but without BLA. Similar results were obtained when the maximum aggregation at 3 minutes was used as an index of aggregation instead of slope of aggregation.](image-url)

**Table 1. Effect of Antibiotics on Platelet Aggregation Response**

<table>
<thead>
<tr>
<th>Agonist/dose</th>
<th>Albumin (%)</th>
<th>Antibiotic</th>
<th>Aggregation Rate (% control)</th>
<th>Extent (% control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thrombin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.08 U/mL</td>
<td>1.8</td>
<td>4 mg/mL</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.08 U/mL</td>
<td>2.7</td>
<td>4 mg/mL</td>
<td>50</td>
<td>14</td>
</tr>
<tr>
<td>0.20 U/mL</td>
<td>1.8</td>
<td>4 mg/mL</td>
<td>70</td>
<td>94</td>
</tr>
<tr>
<td>0.20 U/mL</td>
<td>2.7</td>
<td>4 mg/mL</td>
<td>82</td>
<td>103</td>
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<tr>
<td>Collagen</td>
<td>Cefalothin</td>
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</tr>
<tr>
<td>5 μmol/L</td>
<td>2.0</td>
<td>100 μg/mL</td>
<td>88</td>
<td>16</td>
</tr>
<tr>
<td>5 μmol/L</td>
<td>3.0</td>
<td>100 μg/mL</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Epinephrine</td>
<td>Cefalothin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 μmol/L</td>
<td>2.0</td>
<td>100 μg/mL</td>
<td>35</td>
<td>42</td>
</tr>
<tr>
<td>50 μmol/L</td>
<td>4.0</td>
<td>100 μg/mL</td>
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doses of cephalothin, 10-fold in excess of those used therapeutically, were added to gel filtered platelets, all samples showed inhibition of function regardless of albumin level (when ADP [8 μmol/L], collagen [200 μg/mL], or epinephrine were used as agonists).

Aggregation response of platelets from hypoalbuminemic patients. Complete inhibition of platelet aggregation in response to 8 μmol/L ADP was achieved when platelets from four hypoalbuminemic individuals were incubated overnight with 200 μg/mL cephalothin (Fig 2B). Little or no inhibition of ADP aggregation (8 μmol/L) occurred in platelets from six patients with albumin levels greater than 3.0 g/dL (Fig 2A). The inhibition exhibited by the platelets in hypoalbuminemic plasma was partially reversible (25% to 75%) when the albumin levels were increased to 4.0 to 4.8 g/dL by the addition of 25 g/dL human albumin. Representative aggregation curves from a single subject are shown in Fig 3.

When the albumin concentration of PRP from hypoalbuminemic patients (albumin range, 2.6 to 3.0 g/dL; mean, 2.8 g/dL) receiving cephalothin (1 g every 4 hours) was increased to 4.0 to 4.5 g/dL by the addition of human albumin, the platelet aggregation response to 0.5 μmol/L ADP increased (Fig 4A). The maximum slope of platelet aggregation increased from a mean of 0.72 to 1.6 in patients receiving cephalothin when the albumin level was raised from an average of 2.8 g/dL to 4.2 g/dL. In contrast, the slope of aggregation decreased in PRP samples from patients with hypoalbuminemia who were not receiving βLA (Fig 4B). The maximum slope of aggregation decreased from a mean of 1.7 to 1.2 in samples from those individuals not receiving cephalothin. This latter observation is consistent with previous reports of the effect of albumin concentration on platelet aggregation. Similar effects were obtained by using albumin prepared for human use.

Penicillin binding. The binding of [35S]-labeled benzylpenicillin to albumin-free (<0.04 g/dL) gel-filtered platelets is shown on the Scatchard plot in Fig 5. Binding was specific and reversible, as indicated by displacement of radiolabeled penicillin by unlabeled penicillin. Saturation was achieved in albumin-free platelets at 130 pmol/10⁸ platelets. Scatchard plot analysis of these data indicates, under these conditions, approximately 4,800 binding sites per platelet with an apparent dissociation constant of 200 nmol/L and an affinity constant of 5 x 10⁶ L/mol. At a concentration of 54 pmol of benzylpenicillin/10⁸ platelets, binding of penicillin to platelets was increased in samples with lower albumin concentrations, and platelets showed a decrease in binding with increasing albumin concentrations (Fig 6). Similar results were obtained when stabilized albumin prepared for transfusion was substituted for fatty acid-free albumin. Platelets exposed overnight to radiolabeled penicillin continued to show decreased binding of penicillin at higher albumin levels. However, platelets incubated overnight bound less penicillin than those incubated for 15 minutes. This is most likely due to a loss of platelet integrity that occurs after gel filtration and storage in a non–gas-permeable container.

DISCUSSION

Bleeding episodes observed in patients who receive βLAs have been attributed to platelet dysfunction, hypoproteinemia, and the inhibition of fibrin polymerization. Factors that have been implicated in the increased risk of bleeding include thrombocytopenia, uremia, chemother-
ANTIBIOTIC-MEDIATED PLATELET INHIBITION

A

Cephalothin Treated Hypoalbuminemic Patients

B

Untreated Hypoalbuminemic Patients

Fig 4. Platelet aggregation responses to 0.5 μmol/L ADP were tested using PRP obtained from four hypoalbuminemic patients (mean albumin, 2.8 g/dL) receiving cephalothin (1 g every 4 hours) (A) and six hypoalbuminemic patients receiving no antibiotics (B). Samples were split and the albumin levels of one of the pair were adjusted to approximately 4 g/dL; a similar volume of PBS was added to the control sample. Samples of PRP from patients not receiving antibiotics showed decreased aggregation after addition of albumin, a well-described phenomenon, while the aggregation response of PRP from patients receiving PLAs increased.

apy, chronic illness, and malnutrition. The latter two conditions are often accompanied by hypoalbuminemia. Although serum albumin concentration influences the action of many drugs in vivo, no relationship between albumin concentration and BLA-induced platelet dysfunction has been reported.

In this study, the degree of inhibition of aggregation of gel-filtered platelets by BLAs was shown to be inversely related to albumin levels at concentrations below approximately 3.5 g/dL. This inhibition of function parallels the increased binding of radiolabeled penicillin to platelets at declining albumin levels. Platelet aggregation in patients with low albumin levels was inhibited after an overnight incubation with cephalothin (200 μg/mL), while platelets from patients with normal albumin levels exhibited no such inhibition. This inhibition of BLA-treated platelets was partially reversible after albumin levels were returned to normal by the addition of exogenous albumin.

In this study, platelets bound to the antibiotic in a specific manner and the amount of binding was inversely related to the albumin concentration. The protective effect of albumin is most likely a function of the avid binding of antibiotics to albumin and the consequent depletion of free BLA available to the platelets. The binding of albumin to the antibiotic is reversible and drug molecules are in constant equilibrium between the bound and unbound state. For penicillin, one molecule of drug binds to one molecule of albumin. When concentrations of drug exceed the amounts that can be bound to albumin, or when concentrations of albumin decline, the levels of free unbound drug increase. Although it is clear that antibiotic bound to albumin has no antimicrobial activity, the relationship of protein binding to other drug-related actions has not been explored. All BLAs used in this study

Fig 5. The binding of [14C]-benzylpenicillin to samples of gel-filtered, albumin-free platelets from two donors was determined as previously described for concentrations of benzylpenicillin of 27 pmol through 162 pmol/10^6 platelets. Saturation was previously determined to have been achieved at 130 pmol/10^6 platelets. A Scatchard analysis is presented. Nonspecific binding was determined by the addition of 1,000-fold excess unlabeled benzylpenicillin. A total of 4,800 sites per platelet were seen with an apparent dissociation constant of 200 nmol/L and an affinity constant of 5 x 10^4 mol/L.

Fig 6. The effect of albumin concentration on the binding of [14C]-benzylpenicillin was measured for three different gel-filtered plateletpheresis concentrates. Binding of benzylpenicillin at each albumin level was measured in quadruplicate for each sample using a concentration of 54 pmol of benzylpenicillin/10^6 platelets. Results were averaged and plotted using linear regression analysis. The error bars represent the standard error of the mean.
exhibit significant binding to protein,25,26 and the inhibition of platelet function by each βLA was blocked by albumin in a similar manner.

It is likely that βLAs exert their inhibitory effect by interfering with agonist interaction with platelet surface receptors.12-15 This mechanism is supported by evidence that binding of an α-adrenergic antagonist, (3H)dihydroergocryptine, to platelet adrenergic receptors is reduced twofold, and (3H)serotonin release is completely blocked after incubation of platelets with βLAs.17 Evidence supporting reversible βLA binding to the platelet surface can be found in reports that platelet function returns to normal after exposed platelets are resuspended in βLA-free plasma.13,15 In our storage studies, in which platelets were stored under standard blood banking conditions, but in the presence of a therapeutic concentration of cephalothin, antibiotic-mediated platelet dysfunction was at least partially reversed by the addition of albumin. However, irreversible binding of radiolabeled penicillin to platelets and irreversible inhibition of platelet function after prolonged exposure to very high doses of penicillin (10 to 20 mmol/L) have been reported.12 Using similar conditions, we found that 24 hours of exposure to a high dose of penicillin (5.4 mmol/L) in the presence of lowered albumin levels (1.27 to 1.45 g/dL; average, 1.36 g/L) impaired the aggregation response of platelets to thrombin even after removal of the antibiotic by washing, but no structural changes were observed in glycoprotein Ib or IIb by polyacrylamide gel electrophoresis and immunoblotting (data not shown). This finding is consistent with the report that in rat platelets treated with a variety of βLAs, the loss of platelet function does not correlate with structural changes in surface glycoproteins.15 This irreversible inhibition of platelet function seen at higher doses of penicillin probably is caused by a different mechanism of platelet membrane damage from the one responsible for the reversible inhibition seen at lower doses.

Our observation that inhibition of platelet aggregation by βLAs is inversely related to the albumin level suggests that albumin-bound antibiotic is unable to bind to platelets, or does so with less affinity than does unbound antibiotic. While our experiments do not directly address βLA-induced bleeding, we suspect that the increased frequency of bleeding reported in individuals with chronic illness who are treated with βLAs may be related to low albumin concentration. Our observations may further explain why βLA administration to normal subjects is rarely associated with platelet dysfunction or bleeding.

REFERENCES

Effect of albumin on the inhibition of platelet aggregation by beta-lactam antibiotics

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