Effect of Albumin on the Inhibition of Platelet Aggregation by β-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 β-lactam antibiotics (βLAs), although clinical bleeding most frequently occurs in chronically ill, malnourished patients. Although most βLAs bind to serum albumin, the relative influence of bound versus unbound βLAs on platelet function is unknown. We examined the effect of βLAs on the aggregation of gel-filtered platelets from normal subjects and on platelet-rich plasma (PRP) from hypoalbuminemic patients. Therapeutic concentrations of five βLAs were added to normal platelets at different albumin concentrations (1.5 to 4.5 g/dL). Inhibition of aggregation by the βLAs 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 patients with normal albumin levels and from hypoalbuminemic patients. In addition, we used [35S]-benzylpenicillin to study βLA 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 (Penval 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 levels (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 µg/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 βLA 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 βLAs. All patients and normal donors denied ingestion of aspirin, other nonsteroidal anti-inflammatory drugs, 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 160xg for 15 minutes at room temperature.

Measurement of the aggregation response of normal platelets treated with βLAs. Platelet concentrates obtained from normal individuals were gel-filtered on Sepharose 2B (Pharmacia Fine Chemicals, Piscataway, NJ), as previously described,17 and eluted with 3.8 mmol/L HEPEPS 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, NaH₂PO₄ 3.8 mmol/L. Samples were adjusted to a platelet count of 2 x 10⁹/µL (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,19 was less than 0.4 g/dL. Sam-

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Antibiotic-mediated platelet inhibition

Platelet 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 \( \mu \)g/mL; moxalactam, 400 \( \mu \)g/mL; cefamandole, 13 \( \mu \)g/mL (Eli Lilly); ceftriaxone, 250 \( \mu \)g/mL (Roche Pharmaceutical, Nutley, NJ); and penicillin G 10 \( \mu \)g/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 \( \mu \)L of 80 \( \mu \)mol/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 \( \times 10^{11} \) platelets/mL. Platelet aggregation in response to 8 \( \mu \)mol/L ADP was calculated for samples tested in duplicate.

In separate experiments, PRP from 10 hypoalbuminemic patients (four of whom were receiving cephalothin) 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 \( \mu \)mol/L ADP was measured as described above. The maximum slope of aggregation in response to 8 \( \mu \)mol/L ADP was calculated for samples tested in duplicate.

A 15- \( \mu \)L suspension of Apipezon oil A (Biddle Co, Bluebell, PA) and n-butyl phthalate (1:9 vol/vol) together with 4 \( \mu \)L of \([35S]\)-benzylpenicillin (0.28 to 4.0 nmol/L, 27 to 162 pmol/10\(^7\) platelets; Du Pont Co, Wilmington, DE) in ethanol (final concentration, <0.5\%) was added to 200 \( \mu \)L samples of gel-filtered albumin-free platelets obtained from normal individuals. \(^{39}\) 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 \( \mu \)L of 1% sodium dodecyl sulfate. Radioactivity was measured after addition of Aquasol (Du Pont Co). Binding specificity was assessed by measuring the displacement of \([35S]\)-benzylpenicillin by 1,000-fold excess nonradioactive penicillin. Binding experiments were also performed on three gel-filtered platelet samples 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 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 \( \mu \)mol ADP was used as an agonist. The mean inhibition of aggregation for cephalothin in the five different platelet samples was 64% \( \pm \) 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 \( \mu \)mol/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).

<table>
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<th>Antibiotic</th>
<th>Aggregation Rate (% control)</th>
<th>Extent (% control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cephalothin</td>
<td>88</td>
<td>16</td>
</tr>
<tr>
<td>Penicillin</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
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Table 1. Effect of Antibiotics on Platelet Aggregation Response
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, hypoprothrombinemia, 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

Fig 4. Platelet aggregation responses to 0.5 μmol/L ADP were tested using PRP obtained from four hypoalbuminemic patients (mean albumin, 2.9 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 PIAs increased.

Fig 5. The binding of [35S]-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 a 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 L/mol.

Fig 6. The effect of albumin concentration on the binding of [35S]-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.
interfering with agonist interaction with platelet surface
receptors. This mechanism is supported by evidence that
binding of an α-adrenergic antagonist, \( ^{3} \text{H} \) dithydroergocryptine, to platelet adrenergic receptors is reduced twofold, and \( ^{14} \text{C} \) serotonin release is completely blocked after
incubation of platelets with β-LAs. Evidence supporting
reversible β-LA binding to the platelet surface can be found in
reports that platelet function returns to normal after
exposed platelets are resuspended in β-LA-free plasma. In
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 inhibi-
tion of platelet function after prolonged exposure to very
high doses of penicillin (10 to 20 mmol/L) have been
reported. 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 glyco-
protein 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
β-LAs, the loss of platelet function does not correlate with
structural changes in surface glycoproteins. This irrever-
sible 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
β-LAs 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 β-LA-
induced bleeding, we suspect that the increased frequency
of bleeding reported in individuals with chronic illness who
are treated with β-LAs may be related to low albumin
concentration. Our observations may further explain why
β-LA administration to normal subjects is rarely associated
with platelet dysfunction or bleeding.

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