Inhibition of Ristocetin-induced Platelet Agglutination by Vancomycin

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Ristocetin and vancomycin are structurally similar glycopeptide antibiotics. Both vancomycin and ristocetin in high concentrations (3.0 mg/ml) cause the precipitation of fibrinogen, plasminogen, and IgG from platelet-poor plasma (PPP). In contrast to ristocetin, vancomycin (0.5–1.5 mg/ml) does not agglutinate platelets in normal platelet-rich plasma (PRP) or formalin-treated platelets in the presence of normal PPP. Preincubation of vancomycin (0.5–1.25 mg/ml) with normal PRP, von Willebrand platelets in normal PPP, or formalinized platelets results in inhibition of platelet agglutination induced by ristocetin (0.7–1.25 mg/ml) or ristocetin and normal PPP. This inhibition can be overcome by increasing the final concentration of ristocetin in the platelet suspension. Preincubation of formalin-treated platelets with the major fraction obtained by carboxymethyl-Sephadex C-50 chromatography of commercial vancomycin also results in inhibition of agglutination induced by ristocetin and normal PPP. Incubation with vancomycin (1.25 mg/ml) does not interfere with von Willebrand factor (vWF) or factor VIII coagulant activities in normal PPP or in Sepharose 4B void volume fractions of PPP. These results indicate that vancomycin interacts with normal, von Willebrand, and formalin-treated platelets and inhibits the binding of ristocetin (or ristocetin-vWF complexes).

RISTOCETIN AND VANCOMYCIN are glycopeptide antibiotics with similar structures and bactericidal mechanisms. Both antibiotics contain amino, phenolic, and sugar residues, form ionic bonds with C-terminal carboxyl groups in bacterial cell wall glycopeptide precursors, and cause the precipitation of fibrinogen and other plasma proteins. Ristocetin induces the agglutination of platelets in normal platelet-rich plasma (PRP) and the agglutination of formalin-treated platelets in the presence of the factor VIII-associated von Willebrand factor (vWF). vWF is present in normal plasma, but is absent or reduced in the plasma of most patients with the von Willebrand syndrome. It has been suggested that ristocetin may attach to platelet membrane sulfhydryl or carboxyl groups, alter platelet surface charge, and increase platelet binding affinity for vWF.

Vancomycin does not agglutinate platelets in normal PRP and preliminary reports have indicated that it interferes with ristocetin-induced agglutination. The inhibitory effect might result from the interaction of vancomycin with plasma factor VIII molecules or competition for platelet ristocetin-binding sites. The effect of vancomycin on ristocetin-induced agglutination of normal and formalin-treated platelets is described in this report.
MATERIALS AND METHODS

Blood from 12 normal donors and 3 patients with the von Willebrand syndrome (clinical status: 2 severely affected, 1 mildly affected) was anticoagulated with 1/10 volume 3.8% sodium citrate, and PRP was prepared by centrifugation at 150 g for 10 min. PPP was obtained by centrifuging PRP at 10,800 g for 15 min. Platelets were adjusted to 200,000/μl with PPP, as determined by the Coulter Counter (Model ZBI, Coulter Electronics, Hialeah, Fla.) and phase microscopy, and aggregation studies were performed with collagen, adenosine diphosphate (ADP), and epinephrine as described previously. Stock solutions of ristocetin (Abbott Laboratories, North Chicago, Ill.) and vancomycin (Eli Lilly, Indianapolis, Ind.) were prepared in deionized water or in phosphate (16 mM)-buffered or imidazole (16 mM)-buffered NaCl (0.124 M), pH 7.4. For direct agglutination studies, 50 μl of ristocetin, vancomycin, water, or buffered NaCl (as controls) was added to 0.4-ml samples of either PRP or formalin-treated platelets suspended in phosphate-buffered NaCl (platelets, 300,000/μl) to which 50 μl of normal PPP had been added. For inhibition studies, 50 μl of vancomycin, water, or buffered NaCl (as controls) was incubated for 5 min at 37°C with either 0.4 ml PRP or formalinized platelets, and then ristocetin (or other aggregating agents in the case of PRP) was added to produce the desired final concentrations. The pH of 15-mg/ml stock solutions in water was 6.2 for ristocetin and 3.6 for vancomycin; it was 6.8 for stock solutions of both vancomycin and ristocetin in buffered NaCl. The pH of PRP samples remained unchanged (at 7.8) following the addition of vancomycin or ristocetin.

Proteolytic activity has been found in some ristocetin preparations. For detection of any contaminating proteolytic activity in 2-mg/ml solutions of vancomycin and ristocetin, fibrin polymer clot lysis tests were performed as described by Jenkins et al., and the electrophoretic mobility of fibrinogen α, β, and γ chains was determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis after incubation of 5 mg/ml human fibrinogen (Merck, Sharp, and Dohme, West Point, Pa.) with vancomycin, ristocetin, or water (as control) for either 30 min or 4 hr at 37°C. The electrophoretic mobility of α, β, and γ chains of fibrinogen preincubated with vancomycin and ristocetin was compared with the migration of α, β, and γ chains of human fibrinogen purified from fibrinogen concentrates (Cutter Laboratories, Berkeley, Calif.) by DEAE-cellulose chromatography.

To determine the plasma protein precipitation pattern at high antibiotic concentrations, 30 mg/ml final concentrations of commercial vancomycin or ristocetin were added to 3.0-ml samples of normal PPP and incubated at 37°C for 15 min; the precipitates were collected by centrifugation at 50,000 g for 10 min. Precipitates were suspended in 5 ml Tris-buffered NaCl, pH 7.4, precipitated three times by 3.0 mg/ml vancomycin or ristocetin, and resuspended in 0.5 ml Tris-buffered NaCl for analysis by immunodiffusion and SDS polyacrylamide gel electrophoresis. Double immunodiffusion was performed by the method of Ouchterlony in 1.25% agarose plates using antisera to fibrinogen, albumin, IgG, IgA, IgM, C3 (β1A/β1C), transferrin, ceruloplasmin (Meloy Laboratories, Springfield, Va.), plasminogen, factor VIII, haptoglobin, and hemopexin (Behring Diagnostics, Somerville, N.J.). Electrophoresis on SDS polyacrylamide gels (3%, stacking and 7%, separating gels) was at 3 mAmp/gel in 2 mM Tris HCl, 0.19 M glycine, and 0.1%, SDS, pH 8.3.

To study the effect of vancomycin (1.25 mg/ml final concentration) on vWF and factor VIII coagulant activities, 3-ml samples of normal donor PPP were incubated for 5 min at 37°C with 0.3-mI aliquots of vancomycin (12.5 mg/ml) or water (as control). Then 2-ml samples were fractionated on Sepharose 4B (Pharmacia, Piscataway, N.J.) columns with dimensions of 0.9 × 30 cm, bed volumes of 18 ml, and flow rates of 1.5 2.0 ml/min. Column equilibration and elution were done with 20 mM Tris HCl, 1 mM sodium citrate, and 120 mM NaCl. Fractions of 1.3 ml were collected. Void volumes were determined by passing 0.4-ml solutions of Blue Dextran 2000 (Pharmacia) through the columns. Assays of vWF activity in PPP and column fractions were done by measuring the rate and extent of ristocetin-induced formalinized platelet agglutination by the method of Macfarlane et al. (0.4 ml platelets at 300,000/μl + 50 μl test sample + 50 μl of 10 mg/ml ristocetin). Samples of PPP and column fractions were tested for factor VIII coagulant activity using factor VIII deficient plasma (Dade Reagents, Miami, Fla.) and the method of Bowie et al. based on the one-stage activated partial thromboplastin time. Both vWF and factor VIII activities were expressed in U/ml (100% activity = 1 U/ml; 50% activity = 0.5 U/ml, etc.) and in U/mg protein. Protein concentrations were determined for PPP samples and column fractions by the fluorescamine assay method, which is sensitive in the microgram range.
Commercial vancomycin (200 mg) was fractionated on carboxymethyl-Sephadex (CM-Sephadex C-50, Pharmacia) by discontinuous ammonium acetate elution. Column dimensions were 3 x 50 cm, and 5 g of CM-Sephadex C-50 were used. Fractions of 5 ml were collected and the absorbance at 280 nm (A280) determined. (In order to convert A280 values to mg/ml, it was assumed that the molar absorbance at 280 nm was equivalent for each vancomycin fraction.) Before determining the effects of column fractions on platelet agglutination, ammonium acetate was removed using the ethanol-acetone precipitation method of Nieto and Perkins.

RESULTS

Vancomycin Inhibition of Ristocetin-induced Platelet Agglutination

Vancomycin in concentrations of 1.0, 1.25, and 1.5 mg/ml did not agglutinate platelets in normal PRP. A comparison of platelet agglutination patterns obtained with 1.25 mg/ml ristocetin and 1.25 mg/ml vancomycin is shown in Fig. 1A. Preincubation of PRP from normal donors with vancomycin (0.75-1.25 mg/ml) resulted in inhibition of platelet agglutination induced by 1.25 mg/ml ristocetin (Fig. 1B). This inhibitory effect could be overcome partially by increasing the ristocetin concentration to 1.5 mg/ml (Fig. 1C). When the agglutination response to ristocetin was restored to the platelets of all three von Willebrand patients by the addition of normal PPP, ristocetin-induced agglutination could be inhibited by 1.0-1.25 mg/ml vancomycin. The man with severe von Willebrand syndrome (whose platelet agglutination pattern is shown in Fig. 2) had template bleeding times of 12-16 min, decreased glass bead retention, 26° factor VIII coagulant activity, and no detectable vWF activity.

Vancomycin did not agglutinate formalin-treated platelets suspended in normal PPP (Fig. 3). Preincubation of formalized platelets with vancomycin resulted in inhibition of platelet agglutination induced by ristocetin and PPP. In contrast, preincubation of vancomycin with normal PPP did not interfere with ristocetin-induced agglutination of formalized platelets (Fig. 4). When formalin-treated platelets were incubated with vancomycin (10 mg/ml), centrifuged for 7 min at 1500 g, washed once in 2 ml of phosphate-buffered NaCl and resuspended in 0.4 ml of the same buffer, then agglutination induced by ristocetin (50 μl; 0.7 mg/ml final concentration) and PPP (50 μl) was inhibited. This

![Graph](image-url)
Antibiotic-Induced Colitis
inhibition was reversed completely by increasing the final ristocetin concentration to 1.25 mg/ml. Vancomycin inhibited PPP-induced agglutination of formalinized platelets which had been incubated previously with ristocetin (Fig. 5).

Commercial vancomycin was separated by chromatography on carboxymethyl (CM)-Sephadex C-50 into three peaks and one "shoulder" (Fig. 6A), which were designated fractions I–IV (according to Nieto and Perkins\textsuperscript{22}). Of the 200 mg of vancomycin applied to the column, 166 mg (86\%) was recovered. Most (142 mg) was in fraction IV. Fractions II and III, which may be breakdown products of fraction IV,\textsuperscript{22} were present in small amounts. Insufficient quantities of either fraction I or fraction III were obtained to assess the effects of final concentrations greater than 0.2–0.3 mg/ml on formalinized platelet agglutination. The effects of fractions II and IV on formalinized platelet agglutination induced by ristocetin and normal PPP are shown in Fig. 6B. Preincubation of formalin-treated platelets with 50-\mu l aliquots of concentrated solutions (5 and 8 mg/ml) prepared from either fraction II or fraction IV resulted in inhibition of platelet agglutination. In Fig. 6B this inhibition is compared to that produced by equivalent concentrations of commercial vancomycin.

The PRP from each of 12 normal donors was obtained on 1–3 different days, preincubated with 1.25 mg/ml vancomycin, and tested with aggregating agents. In PRP samples from 2 donors the amplitude of aggregation responses to collagen (0.3 mg/ml) was decreased by 30\%–35\% and primary aggregation in response to $10^{-6}\text{ M ADP}$ was reduced by 30\%. Aggregation was normal in these two donors when the final concentration of ADP was increased to $10^{-5}\text{ M}$. In PRP obtained from two other donors, there was a 22\%–35\% inhibition of primary aggregation induced by $10^{-5}\text{ M ADP}$. However, this inhibitory effect of vancomycin was not found consistently when the PRP of these two donors

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![Fig. 6. Effects of commercial vancomycin and purified vancomycin fractions on ristocetin-induced agglutination of formalin-treated platelets in the presence of normal PPP. (A) Purification of 200 mg commercial vancomycin on CM-Sephadex C-50. A\textsubscript{280} absorbance at 280 nm. Fraction volume was 5 ml. (B) Inhibitory effects of commercial vancomycin and column fractions II and IV. Before addition of 50 \mu l of ristocetin (R; 1.0 mg/ml final concentration) and 50 \mu l of normal PPP, formalinized platelets were incubated at 37\°C for 3 min with either 50 \mu l of phosphate-buffered saline (R), commercial vancomycin in final concentrations of 0.5 and 0.8 mg/ml (R + V), column fraction II in final concentrations of 0.5 and 0.8 mg/ml (R + II), or column fraction IV in final concentrations of 0.5 and 0.8 mg/ml (R + IV).](image)
was tested with $10^{-5}\ M$ ADP on different days. Vancomycin did not inhibit either primary or secondary aggregation induced by epinephrine ($4.5 \times 10^{-5}\ M$).

**Precipitation of Plasma Proteins by Ristocetin and Vancomycin**

Both ristocetin and vancomycin (3.0 mg/ml) caused the precipitation of fibrinogen and other proteins from normal PPP (Fig. 7). By double immunodiffusion these other proteins included plasminogen and IgG, but not albumin, IgA, IgM, C3 ($\beta 1A/\beta 1C$), haptoglobin, hemopexin, transferrin, ceruloplasmin, or factor VIII.

At concentrations of 2 mg/ml neither vancomycin nor ristocetin caused proteolysis of fibrin polymer clots or altered the electrophoretic mobility of fibrinogen $\alpha$, $\beta$, or $\gamma$ chains in SDS-polyacrylamide gels.

**Effects of Vancomycin on Plasma Factor VIII Coagulant and vWF Activities**

Normal PPP samples were incubated with vancomycin (1.25 mg/ml final concentration), and 50 $\mu$l of vancomycin-treated PPP was tested in vWF assays using 0.4 ml formalized platelets and 50 $\mu$l of 10 mg/ml ristocetin. There was no inhibition by 1.25 mg/ml vancomycin of normal PPP vWF activity. Following incubation of normal PPP samples with vancomycin (1.25 mg/ml final concentration), vWF and factor VIII coagulant activities were separated by Sepharose 4B chromatography. There was no inhibition by vancomycin of vWF or factor VIII coagulant activities in the void volume fractions of normal PPP, irrespective of whether activities were expressed in U/ml (as in the representative experiments illustrated in Fig. 8) or in U/mg protein.

**DISCUSSION**

Vancomycin and ristocetin are structurally similar antibiotics$^{1,2}$ which, in high concentrations, cause the precipitation of plasma fibrinogen, plasminogen, IgG,
Fig. 8. vWF (A) and factor VIII coagulant activities (B) in Sepharose 4B fractions of normal PPP which had been preincubated either with a final concentration of 1.25 mg/ml of vancomycin (Vanco) or with water (as control). A 280: absorbance at 280 nm. V₀: void volume. Detection of albumin and plasminogen was by double immunodiffusion. Detection of fibrinogen was by double immunodiffusion and SDS-polyacrylamide gel electrophoresis.
and other proteins (Fig. 6). Ristocetin induces the agglutination of platelets in normal PRP and of formalin-treated platelets in the presence of normal PPP. In contrast, vancomycin inhibits ristocetin-induced platelet agglutination in PRP and in suspensions of formalinized platelets—an effect which cannot be the result of alterations in platelet metabolism. Fraction IV (the major peak obtained by cation-exchange chromatography of commercial vancomycin) and one of the minor fractions (II) are both potent inhibitors of platelet agglutination induced by ristocetin and normal PPP.

Partial reversal of the vancomycin inhibitory effect is obtained by increasing the concentration of ristocetin added either to normal PRP or to formalinized platelets in the presence of normal PPP. Additionally, aggregation of platelets in PRP by collagen, ADP, and epinephrine is not impaired significantly by preincubation of PRP by vancomycin. These results indicate that vancomycin inhibition of ristocetin-induced agglutination is relatively specific.

Agglutination is inhibited when formalin-treated platelets are incubated with vancomycin and then sedimented, washed, and resuspended in buffered NaCl prior to the addition of ristocetin and PPP. This inhibition is reversed by increasing the final concentration of ristocetin. Platelet agglutination in response to ristocetin and PPP is reduced if vancomycin is added after the incubation of formalin-treated platelets with ristocetin. These findings suggest that vancomycin and ristocetin compete for the same platelet receptor sites. In previous studies, Coller et al. could not detect a stable interaction between 125I-vancomycin and platelets separated in void volume fractions of Sepharose 2B. It may be that vancomycin is easily displaced from platelet binding sites. Alternatively, iodinating a phenol group in vancomycin may interfere with binding of the antibiotic to platelets.

Platelet agglutination is not inhibited when vancomycin is incubated with PPP prior to the addition of PPP to a ristocetin-containing suspension of formalinized platelets. Furthermore, vWF and factor VIII coagulant activities in PPP and in Sepharose 4B column fractions of PPP were not reduced by prior incubation with vancomycin. Thus, a direct effect of vancomycin on factor VIII-associated vWF activity does not account for inhibition of ristocetin-induced platelet agglutination.

vWF is present in the membrane and granular fractions of normal human platelets, but cannot be detected in platelets from patients with severe von Willebrand syndrome. When the latter platelets are incubated in normal plasma and then agglutinated by ristocetin, vWF from the plasma binds to the clumped platelets. It is clear that platelet agglutination induced by ristocetin and vWF does not require Ca or active platelet metabolism. However, it is not yet known if ristocetin binds to platelets and induces the attachment of vWF, or if complexes of ristocetin and vWF attach to platelets and result in agglutination.

Externally exposed platelet surface proteins may be receptor sites for ristocetin or complexes of ristocetin and vWF. Evidence has been presented that platelet membrane "glycoprotein I" (molecular weight 155,000) and a protein of similar size which is loosely associated with the outer platelet membrane ("glycocalcin," molecular weight 148,000) are important in agglutination.
induced by ristocetin and vWF. Vancomycin may interfere either with the binding of structurally similar ristocetin molecules or with the binding of ristocetin–vWF complexes to these (or other) proteins on platelet surfaces. Our finding that vancomycin interferes with the ristocetin-induced agglutination of platelets, from severely affected von Willebrand patients, suspended in normal PPP is evidence that vancomycin does not inhibit the interaction of ristocetin with vWF which is already associated with platelet surfaces. Vancomycin should be useful in further studies of the interaction of platelets with ristocetin and vWF.

ACKNOWLEDGMENT

We appreciate the excellent technical assistance of Elsie Kwok and Joseph Troll.

REFERENCES


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