Effects of Penicillin G on Platelet Aggregation, Release, and Adherence to Collagen[1] (36980)

J.-P. CAZENAVE,[2] M. A. PACKHAM, M. A. GUCCIONE, AND J. F. MUSTARD

Department of Biochemistry, University of Toronto, Toronto, Ontario, and Department of Pathology, Faculty of Medicine, McMaster University, Hamilton, Ontario, Canada

In 1947 Fleming and Fish (1) observed that impure penicillin (6250 units/ml) interfered strongly with coagulation and clot retraction *in vitro*. With pure penicillin, clot retraction was impaired with as little as 340 units/ml. They pointed out that such concentrations of penicillin might be achieved upon local administration of the antibiotic during bone surgery or after tooth extraction.

Although platelet functions are affected by many drugs (2), there are very few reports concerning the effects of antibiotics on platelets, except for toxic or allergic thrombocytopenic effects (3). McClure *et al.* (4) showed that carbenicillin, in large doses, impairs ADP-induced platelet aggregation and they concluded that this impairment was responsible for the acquired bleeding disorder they observed in their patients. Other investigators have also reported bleeding associated with carbenicillin therapy (5, 6).

Penicillin has been shown to coat red blood cells (7) and there are reports in the literature of hemolytic anemia (8), granulopenia (9) and thrombocytopenia (10) following intensive therapy with penicillin or its analogues, attributed to immunologic reactions.

This study reports investigations of the effects of penicillin G on some *in vitro* reactions of human platelets in platelet-rich plasma and of rabbit and pig platelets in suspensions of washed platelets.

Materials and Methods. Reagents. ADP (Sigma Chemical Co., St. Louis, MO) and bovine thrombin (Parke Davis and Co., Detroit, MI) were diluted with modified Tyrode's solution [no calcium or magnesium (9)]. Collagen (bovine tendon, Sigma) was used as a suspension in modified Tyrode's solution (11) or as acid soluble collagen (0.25% in 0.522 *M* acetic acid); for aggregation studies the latter was diluted with 0.85% NaCl solution and the dilutions used are indicated in the tables. Penicillin G, USP-sodium, 1650 units/mg, (General Biochemical, Chagrin Falls, OH) was prepared just before use as a stock solution of 88,000 units/ml (0.148 *M*) in distilled water; modified Tyrode's solution was used for dilution. Carbenicillin, disodium salt (Ayerst Laboratories, Montreal, Quebec) was prepared in the same way and at the same molar concentration as penicillin.

Preparation of human platelet-rich plasma (PRP). Blood was obtained from subjects who had not taken any drugs for at least 10 days (except for the subject receiving acetylsalicylic acid (ASA)). The anticoagulant used was 3.8% trisodium citrate dihydrate, 1 part to 9 parts of blood. PRP was prepared by centrifugation at 77*g* for 15 min at room temperature (11).

Preparation of suspensions of washed platelets. Suspensions of twice-washed platelets from rabbits (12), pigs, or humans (13) were prepared as described previously except that for pig platelets the osmolarity of the washing and suspending solutions was adjusted to 340 mOsm with 30% NaCl. Platelets from all species were finally resuspended at a platelet count of 700,000/mm^3 in Tyrode's solution containing 0.35% bovine albumin (Pentex, Miles Laboratories, Kankakee, IL); with pig and human platelets apyrase was also included in the suspending media and the suspensions were stored at 37° (13).

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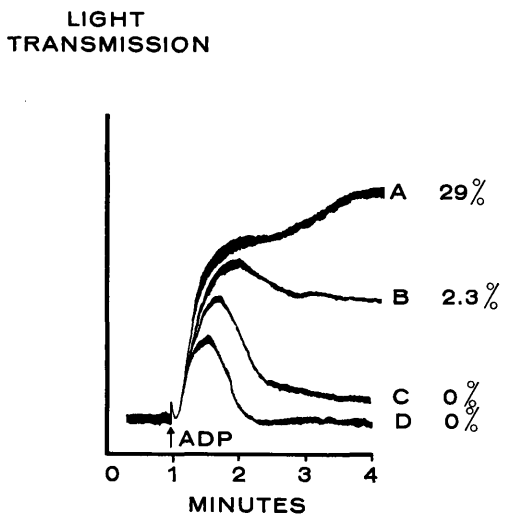

FIG. 1. The effect of penicillin G on ADP-induced platelet aggregation in human citrated PRP. Increased light transmission indicates aggregation. PRP was incubated at 37° for 10 min with: A. Modified Tyrode's solution, B. Penicillin G (1000 units/ml), C. Penicillin G (4000 units/ml), and D. Penicillin G (8000 units/ml) before the addition of ADP (2.1 μM) at the point indicated by the arrow. The percent of release of radioactivity from the ^{14}C-serotonin-labeled platelets is shown beside each curve.

Platelet labeling. Platelets were labeled with ^{14}C-serotonin (5-hydroxytryptamine-3′-^{14}C-creatinine sulphate, 55 μCi/μmole (Amersham/Searle, Arlington Heights, IL) in PRP or in the first washing fluid (0.06 μCi/ml) (14). Pig platelets in the first washing fluid were labeled with ^{51}Cr (sodium chromate, 1 μCi/μl, Amersham/Searle) by incubation at 37° for 20 min with 10 μCi/ml of washing fluid. They were washed twice more before final resuspension. Labeling of pig platelets with ^{3}H-DFP [(di-isopropyl-(1,3-H^{3}) phosphorofluoridate, 3.3 Ci/mmole, Amersham/Searle)] was done in the same way by incubation for 5 min with 0.25 μCi/ml of washing fluid.

Platelet aggregation was studied at 37° by a turbidimetric method (15). One milliliter of PRP or platelet suspension was incubated with 0.1 ml of antibiotic solution (or modified Tyrode's solution in the control) for 10 min before the addition of 0.1 ml of aggregating agent. All concentrations given are the final concentrations after the addition of the antibiotic.

Radioactivity in the ambient fluid 3 min after the addition of an aggregating agent was determined as described previously (16). A liquid scintillation counter was used for ^{14}C and ^{3}H and a well-type gamma scintillation crystal counter for ^{51}Cr. Released or freed radioactivity was calculated as a percentage of the radioactivity in the platelets; the small amount of radioactivity in the ambient fluid of unstimulated platelets was subtracted before this calculation was made.

Platelet adherence to collagen-coated tubes. Pyrex glass tubes (10 × 75 mm) were washed in detergent, soaked in 1 *N* NaOH, rinsed, soaked in 1 *N* HCl, rinsed thoroughly and dried. Acid-soluble collagen (1 ml) was added to each tube, the tubes were covered with parafilm and rotated end-over-end at 15 rpm for 10 min at 22°. The solution was decanted and the tubes rinsed by filling them 4 times with modified Tyrode's solution. Ten minutes before the platelet suspension was to be added to the collagen-coated tube, penicillin G (0.1 ml/ml of suspension) or modified Tyrode's solution in control experiments, was added to the platelets and incubated at 37°.

One milliliter of platelet suspension containing penicillin G (or modified Tyrode's solution) was added to the coated tube and was then covered with parafilm and rotated end-over-end at 15 rpm for 10 min at 22°. Before and after rotation a sample of the platelet suspension was examined by phase contrast microscopy to ensure that no aggregation had occurred.

To measure the amount of radioactivity released or freed from the platelets and the amount of platelet material adherent to the tubes, the following samples were examined:

a. 0.1 ml of the original suspension

b. 0.1 ml of the supernatant fluid from the original platelet suspension after centrifugation at 12,000*g* for 1.5 min (Eppendorf centrifuge, Brinkmann, Rexdale, Ontario)

c. 0.1 ml of the supernatant fluid from the suspension centrifuged after the rotation process

d. radioactivity adherent to the tubes: the tubes were rinsed four times with modified Tyrode's solution and drained. They were then

TABLE I. The Effect of Penicillin G on Aggregation and on Release of Radioactivity from ^{14}C-serotonin-labeled Human Platelets in PRP Exposed to ADP, Thrombin, or Collagen.

Aggregating agent added to PRP (final concn)	Final concn of penicillin G (units/ml)	Maximum aggregation (% of control)	^{14}C released	
			% of total ^{14}C in platelets	% of control
Subject 1[b]				
ADP 0.8 μM	0[a]	100	35.5	100
	1000	93	33.7	95
	4000	65	1	3
	8000	38	0	0
Collagen[c] 1/2000	0[a]	100	15.3	100
	1000	91	8	52
	4000	52	0	0
	8000	14	0	0
Collagen[c] 1/5000	0[a]	100	14.6	100
	1000	48	0	0
	4000	21	0	0
	8000	12	0	0
Subject 2				
ADP 0.8 μM	0[a]	100	0.12	—
	1000	81	0	—
	4000	70	0	—
	8000	57	0	—
Thrombin 0.4 units/ml	0[a]	100	1.0	—
	1000	92	1.3	—
	4000	68	1.0	—
	8000	37	1.0	—
Subject 3[d]				
ADP 16 μM	0[a]	100	1.4	—
	8000	90	1.4	—
ADP 0.8 μM	0[a]	100	0.8	—
	1000	96	0.5	—
	4000	89	0.7	—
	8000	70	0.6	—
Collagen[c] 1/1000	0[a]	100	5.8	—
	1000	79	6.2	—
	4000	66	4.4	—
	8000	52	3.6	—

[a] Modified Tyrode's solution instead of penicillin G solution added to control samples.

[b] One of three experiments, with different subjects, that gave similar results. Platelet count 300,000/mm^3.

[c] Diluted acid soluble collagen.

[d] Subject 3 was receiving 2.6 g of ASA per day in four equal doses. Platelet count 320,000/mm^3.

treated with 0.5 ml of NCS (Nuclear Chicago Solvent, Amersham/Searle). The NCS was transferred quantitatively to counting vials using 10 ml of toluene-fluor solution. Each value in Table V is the mean of 5 replicates. A typical control value, 23.5%, had a range of 21.6 to 26.2% with S.E. ± 0.9.

Each of the three 0.1 ml samples taken earlier was also dissolved in 0.5 ml of NCS and mixed with toluene-fluor solution for liquid scintillation counting.

Results. Human PRP. Aggregation and release. Penicillin G inhibited aggregation induced by ADP in human PRP; the extent of

TABLE II. Comparison of Effects of Penicillin G and Carbenicillin on Aggregation and on the Release of Radioactivity from ^{14}C-serotonin-labeled Human Platelets in PRP Exposed to ADP, Thrombin, or Collagen.

Aggregating agent (final concn)	Antibiotic (concn incubated with platelets)	Maximum aggregation (% of control)	^{14}C released[a] (% of total ^{14}C in platelets)
ADP, 2.5 μM	0[b]	100	21.1
	Penicillin, 13.4 mM[c]	59	0
	Carbenicillin, 13.4 mM	74	0
	Carbenicillin, 20 mM	59	0
Thrombin, 0.3 units/ml	0[b]	100	48.4
	Penicillin, 13.4 mM	11	0
	Carbenicillin, 13.4 mM	12	1.0
	Carbenicillin, 20 mM	6	0
Collagen	0[b]	100	24.3
	Penicillin, 13.4 mM	5	0
	Carbenicillin, 13.4 mM	77	10.3
Collagen	0[b]	100	13.3
	Penicillin, 1.7 mM	45	1.5
	Carbenicillin, 13.4 mM	59	3.9

[a] With ADP, samples were centrifuged after 4 min for measurement of released radioactivity. In all other experiments, samples were centrifuged after 3 min.

[b] Modified Tyrode solution instead of antibiotic solution added to control samples.

[c] 13.4 mM penicillin G = 8000 units/ml. Platelet count 450,000/mm^3. This is one of two experiments that gave similar results.

inhibition was greater with higher concentrations of penicillin G (Fig. 1). Inhibition was apparent with PRP both from normal subjects and from a subject taking acetyl salicylic acid (ASA), (Table I). The highest concentration of penicillin G used (8000 units/ml) inhibited the primary phase of ADP-induced aggregation in the subject taking ASA. In PRP from untreated subjects, penicillin G inhibited both the first and second phase of ADP-induced aggregation and the accompanying release of radioactivity from ^{14}C-serotonin-labeled platelets (Fig. 1, Table I).

Thrombin-induced aggregation of human platelets in PRP was inhibited by penicillin G (Table I) but the concentration of thrombin used was insufficient to cause release of appreciable amounts of radioactivity from the ^{14}C-serotonin-labeled platelets.

Penicillin G also inhibited aggregation caused by collagen in PRP from both untreated subjects and the ASA-treated subject. At 1000 units/ml it completely blocked release caused by the lowest concentration of collagen used.

ADP- and thrombin-induced aggregation and release were inhibited by both penicillin and carbenicillin (Table II). Comparison of the effects of equimolar concentrations of these antibiotics showed that penicillin reduced the extent of primary ADP-induced aggregation slightly more than did carbenicillin. Penicillin was much more effective than carbenicillin in reducing the extent of aggregation and release caused by low concentrations of acid-soluble collagen (Table II).

Pig and rabbit platelet suspensions. Aggregation and release. Penicillin G strongly inhibited ADP-induced aggregation in suspensions of washed platelets from pigs (Table III) and rabbits (Table IV). In these suspensions, secondary aggregation and release do not occur upon the addition of ADP. Aggregation and release of radioactivity from ^{14}C-serotonin-labeled platelets stimulated with thrombin or collagen was inhibited by penicillin G (Tables III and IV). Inhibition was greater with the higher concentrations of penicillin G and was more apparent when low concentrations of stimuli were used.

Adherence to collagen-coated surface. Penicillin G diminished the extent of adherence of

TABLE III. Effect of Penicillin G on Aggregation and on the Release of Radioactivity from ^{14}C-serotonin-labeled Pig Platelets in Suspension in Tyrode-albumin Exposed to ADP, Thrombin or Collagen.

Aggregating agent added to platelet suspension[a] (final concn)	Final concn of penicillin G (units/ml)	Maximum aggregation (% of control)	^{14}C released	
			% of total ^{14}C in platelets	% of control
Experiment 1				
ADP[a] 3.8 μM	0[b]	100	0	—
	8000	28	0	—
ADP[a] 0.76 μM	0[b]	100	0	—
	800	63	0	—
	1000	50	0	—
	4000	26	0	—
	8000	20	0	—
Thrombin 0.4 units/ml	0[b]	100	81.8	100
	1000	82	22.4	27.4
	8000	37	4.3	5.2
Thrombin 0.2 units/ml	0[b]	100	61.2	100
	1000	84	47.6	77.7
	4000	42	5	8.2
	8000	9	0	0
Collagen[c] 1/500	0[b]	100	14.0	100
	1000	91	10.5	75.4
	4000	64	6.4	45.8
Experiment 2				
ADP[a] 0.76 μM	0[b]	100	0	—
	1000	71	0	—
	4000	41	0	—
	8000	24	0	—
Thrombin 0.4 units/ml	0[b]	100	80.7	100
	1000	96	57.7	71.6
	4000	15	1.5	1.8
	8000	6	0	0
Collagen[d]	0[b]	100	25.5	100
	1000	98	25.3	99.3
	4000	71	16.4	64.5
	8000	45	9.1	35.7

[a] For aggregation studies with ADP, human fibrinogen (0.05%) was included in the platelet suspending medium (13).

[b] Modified Tyrode's solution instead of penicillin G solution added to these control samples.

[c] Diluted acid soluble collagen.

[d] Collagen suspension.

washed human, rabbit, or pig platelets to a collagen-coated surface (Table V). Little release of radioactivity from ^{14}C-serotonin-labeled platelets occurred in these experiments, and no aggregation was observed microscopically. When pig platelets were labeled with ^{51}Cr or ^{3}H-DFP, the same pattern of inhibition of adherence by penicillin G was obtained (Table V).

Discussion. Penicillin G (1000 to 8000 units/ml; 1.7 to 13.4 mM) inhibits both ADP-induced platelet aggregation and the release reaction caused by thrombin or collagen. These effects are evident with platelets from humans, rabbits, and pigs. The results with ADP in human PRP are in agreement with those reported by McClure *et al.* (4) who used carbenicillin, an analogue of penicillin.

TABLE IV. Effect of Penicillin G on Aggregation and on the Release of Radioactivity from ^{14}C-serotonin-labeled Rabbit Platelets in Suspension in Tyrode-albumin exposed to ADP, Thrombin or Collagen.

Aggregating agent added to platelet suspension (final concn)	Final concn of penicillin G (units/ml)	Maximum aggregation (% of control)	^{14}C released	
			% of total ^{14}C in platelets	% of control
ADP 0.8 μM	0[a]	100	0.2	—
	1000	101	0.1	—
	4000	48	0	—
	8000	18	0	—
Thrombin 0.8 units/ml	0[a]	100	30.3	100
	1000	96	24.9	82.4
	4000	59	8.6	28.5
	8000	5	0.7	2.4
Thrombin 0.4 units/ml	0[a]	100	12.0	100
	1000	10	0.9	7.4
	4000	2	0.7	5.6
	8000	0	0.4	3.6
Collagen[b] 1/1000	0[a]	100	46.0	100
	1000	90	40.2	87.4
	4000	75	22.1	48.1
	8000	50	8.9	19.3
Collagen[b] 1/2000	0[a]	100	30.4	100
	1000	89	17.9	59.0
	4000	12	0.8	2.6
	8000	10	0.6	2.1

[a] Modified Tyrode's solution instead of penicillin G solution added to these control samples.
[b] Diluted acid soluble collagen.

McClure *et al.*, however, reported that carbenicillin did not impair release caused by collagen or thrombin, whereas we found that both antibiotics did inhibit release caused by these agents (although carbenicillin was less inhibitory than penicillin). The most likely explanations for this discrepancy are (i) that the concentrations of thrombin and collagen used by McClure *et al.* were probably much higher than those used in the present study and overcame the inhibitory effect that can be demonstrated when low concentrations of these stimuli are used, and (ii) that the concentration (1.7 mM) of carbenicillin they used was much lower.

It is possible that at least part of the inhibition by these antibiotics of the release reaction induced by thrombin or collagen may be related to their inhibition of ADP-induced aggregation because it has been found that extensive release following the addition of low concentrations of thrombin or collagen does not occur until aggregation is well underway (17). Therefore, if aggregation by released ADP is inhibited, the extent of release would also be diminished. The inhibitory effect of penicillin G (or carbenicillin) on ADP-induced secondary aggregation and the accompanying release reaction may be attributable to its inhibition of primary ADP-induced aggregation. It has been shown that secondary aggregation does not occur unless maximum (or near-maximum) primary aggregation has taken place (18).

Inhibition of collagen-induced platelet aggregation and release could be explained on the basis of the observation that penicillin G greatly diminishes the extent of platelet adherence to a collagen-coated surface. This effect was apparent under conditions in which no aggregation and very little release occurred so inhibition of aggregation and release cannot be responsible for penicillin G blocking platelet adherence to collagen. These

TABLE V. Effect of Penicillin G on Adhesion to a Collagen-coated Surface of Washed Pig, Rabbit or Human Platelets labeled with ^{14}C-serotonin, ^{51}Cr, or ^{3}H-DFP.

Species	Platelet label	Final concn of penicillin G (units/ml)	Adhesion: % of total radioactivity in platelet suspension	Adhesion: % of control	Radioactivity in ambient fluid, % of total in platelets
Human	^{14}C-serotonin	0[a]	27.2	100	0.2
		1000	17.8	65.3	1.3
		4000	4.4	16.3	0
		8000	0.9	3.2	0
Rabbit	^{14}C-serotonin	0[a]	16.8	100	6.3
		4000	2.1	12.6	1.7
		8000	0.2	1.2	0.7
Pig[b]	^{14}C-serotonin	0[a]	23.5	100	0.01
		1000	14.5	61.7	0.01
		4000	4.0	17	0.01
		8000	2.1	8.9	0
Pig	^{51}Cr	0[a]	25.2	100	3.4
		1000	13.7	54.3	5.7
		4000	9.7	38.4	5.3
		8000	9.1	36.2	5.4
Pig	^{3}H-DFP	0[a]	28	100	4.7
		8000	9.7	34.6	6.4

[a] Modified Tyrode's solution instead of penicillin G solution added to these control samples.

[b] Typical of 4 experiments that gave similar results.

(Microscopic examination of glass slides coated with acid-soluble collagen showed that penicillin did not alter the amount of collagen on the glass surface).

results were confirmed in experiments with platelets labeled with ^{51}Cr or ^{3}H-DFP in which inhibition of adherence by penicillin G was observed. These labels are not released from the platelets in the same way as ^{14}C-serotonin, nor are they subject to re-uptake if they are lost from the platelets.

Although the mechanism by which penicillin G inhibits platelet aggregation, the release reaction, and platelet adherence to a collagen-coated surface cannot be determined from these experiments, some suggestions can be made on the basis of observations obtained with other types of cells.

It is unlikely that the concentration of penicillin used inhibit platelet metabolism because the concentrations required to inhibit respiration and lactate formation in thin slices of guinea pig cerebral cortex were much higher (20,000 units/ml) than those used in our study (19). According to Few and Schulman (20), penicillin G is not a surface-active agent at the pH and concentrations used.

Penicillin G is known to coat red blood cells *in vitro* (10,000 units/ml) and *in vivo* (12–25 million units/day, intravenously) (7). It seems likely that it may also coat platelets and thus block some of the sites on the platelet surface where ADP, thrombin or collagen exert their effects.

In clinical medicine the plasma concentrations of penicillin G effective in common infections are in the range of 0.1 to 1 unit/ml (21). Therefore, an effect of penicillin G on platelet function does not seem to be relevant in such cases. However, when massive penicillin therapy is given intravenously, concentrations of 1000 units/ml are achieved for long periods of time (22) and the concentration would certainly be much higher immediately following each injection. In these cases, and after local administration [as pointed out by Fleming and Fish (1) in respect to clot retraction], it seems possible that plate-

let function might be impaired. In bacterial endocarditis, microthrombi are thought to be responsible for the renal and cerebral lesions (23), and it seems possible that penicillin, besides its antibacterial role, may also have an antithrombotic effect at the very high doses generally used in this condition.

Summary. Penicillin G (1000–8000 units/ml) inhibits ADP, collagen or thrombin-induced platelet aggregation and the release reaction in human PRP and in suspensions of washed platelets from rabbits or pigs. Adherence of washed platelets from all three species to a collagen-coated surface is also inhibited by penicillin G in this concentration range. Penicillin G may coat platelets, as it does red cells, block the sites where the aggregating agents interact with the platelets, and thus inhibit the response of platelets to them. Since these concentrations are achieved *in vivo* when massive penicillin therapy is used or penicillin is applied locally, these observations may be relevant to alterations of platelet function during such treatment.

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