

Shortening of the Bleeding Time in Rabbits by Hydrocortisone Caused by Inhibition of Prostacyclin Generation by the Vessel Wall

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ABSTRACT The effect of hydrocortisone on thrombocytopenic bleeding has been studied in rabbits using a jugular vein bleeding-time technique and a microvascular bleeding-time technique. An inverse relationship was found between the bleeding time and platelet count with both techniques in rabbits made thrombocytopenic by either X-irradiation or injection of heterologous platelet antiserum. Hydrocortisone shortened both bleeding times in thrombocytopenic animals when given in single large doses intravenously (25–100 mg/kg), in daily doses (6 mg/kg) intramuscularly, and shortened the jugular bleeding time when applied to the outside of the jugular vein or instilled intraluminally into the vein. This effect was also noted in normal animals. The effect on thrombocytopenic bleeding was dose related. When given daily, the effect was greater when hydrocortisone was given for 10 d than for 5 d. Both indomethacin and tranilcypromine also reduced the jugular vein bleeding time when instilled intraluminally into the jugular vein, whereas exogenously provided arachidonic acid reversed the effect of hydrocortisone but did not reverse the effect of indomethacin or tranilcypromine. Exogenously provided linoleic acid did not have any effect. Perfusion of the vessel segment with prostacyclin (PGI_2) reversed the effect of intraluminally administered hydrocortisone, indomethacin, and tranilcypromine. Similarly, hydrocortisone, indomethacin, and tranilcypromine all reduced the rate of loss of fluid from a standard wound in isolated

vessels emptied of blood and perfused with saline under constant pressure. PGI_2 reversed the action of these three agents, however, arachidonic acid reversed only the effect of hydrocortisone and did not reverse the effect of indomethacin and tranilcypromine. The generation of PGI_2 -like material and 6-keto-prostaglandin $\text{F}_{1\alpha}$ from jugular vein strips was prevented by prior exposure of the animals or vessel wall to hydrocortisone. These results are compatible with the hypothesis that the vessel wall releases smooth muscle-relaxing prostaglandins when injured and that inhibition of prostaglandin formation by hydrocortisone enhances hemostasis by allowing vasoconstriction to be maintained.

INTRODUCTION

For years there has been a clinical impression that corticosteroids reduce bleeding in thrombocytopenic patients without necessarily increasing the platelet count (1–4). Confirmation of this impression presents difficulty because it requires that serial bleeding times be measured in a randomized study in thrombocytopenic patients who are carefully matched both for severity of thrombocytopenia and for the nature of the underlying disease. In this paper we report the effects of hydrocortisone on the bleeding time in normal rabbits and in rabbits made thrombocytopenic by irradiation and/or infusion of heterologous platelet antiserum, and demonstrate that hydrocortisone shortens the bleeding time without increasing the platelet count.

Corticosteroids have been shown to inhibit prostaglandin synthesis by inhibiting the deacylation of phospholipid by phospholipase A_2 (5). Evidence is presented which suggests to us that the hemostatic effect of hydrocortisone may be caused in part by inhibition of the formation of smooth muscle-relaxing (prostacyclin

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[PGI₂]-like)¹ prostaglandins by the vessel wall, which are produced as a normal vascular response to injury. This hypothesis is supported by the demonstration that other prostaglandin synthesis inhibitors, namely indomethacin and tranilcypromine, also shorten the bleeding time, that all three agents inhibit the synthesis of PGI₂-like material by the vessel wall, and that the effect of all three agents on the bleeding time is reversed by local perfusion of the vessel with PGI₂.

METHODS

Jugular bleeding time

New Zealand white rabbits were anesthetized with pentobarbital and the jugular veins were isolated and stripped of all adventitial tissue. A right-angled puncture was made in the vessel wall with a 23-gauge needle. The shed blood that flowed from the wound was collected in a trough-shaped stainless steel drain that was positioned beneath the vein at a 45° angle. The drain was continuously irrigated with 0.9% sodium chloride to keep it free of clotted blood and to facilitate measurement of the time taken between the puncture and cessation of bleeding. Care was taken to ensure that the fluid used for irrigation did not come in contact with the jugular vein.

Microvascular bleeding time

The microvascular bleeding time was measured using a standard incision through the ear of New Zealand white rabbits. The ear was warmed for 5 min to 37°C by immersion into a bath that contained 0.9% sodium chloride. A 6-mm full thickness incision was then made through the ear with a scalpel blade. In selecting the site for incision, care was taken to avoid any visible vessels. The incised ear was then immediately reimmersed in the 0.9% saline bath, which was gently agitated with a magnetic stirrer. The time taken for bleeding to cease was recorded.

Production of thrombocytopenia in rabbits

Thrombocytopenia was produced in three ways; irradiation, heterologous platelet antisera, or by a combination of these two techniques.

Irradiation. Thrombocytopenia was produced by irradiating the animals with 930 rads for 30 min with a caesium source. During irradiation, the ears and neck area of the rabbits were shielded with a lead ribbon to avoid damage to the jugular veins and ear vessels. The platelet count began to fall 4 d after irradiation and usually reached a nadir at 10 d.

Heterologous anti-platelet serum. Platelets were isolated from rabbit blood and washed twice with calcium-free Tyrode's buffer (pH 6.2) (6), 3×10^9 platelets were suspended in 3.0 ml of calcium-free Tyrode's buffer (pH 7.35) and disrupted by sonication. This suspension was emulsified with 3.0 ml of complete Freund's adjuvant and injected into deep intramuscular sites in the hind and forelegs of a sheep. Booster doses without adjuvant were given at weekly intervals for 3 wk. The animals were bled 5 d after the final injection. The serum was separated and heat inactivated for 45 min at 56°C

and the denatured protein removed by centrifugation. The potency of the anti-platelet serum was tested, and it was found that severe thrombocytopenia was produced in rabbits by a dose that varied between 0.04 and 0.1 ml/kg among the different batches of antisera used.

Effect of hydrocortisone on the jugular vein and microvascular bleeding times

In all but the series of experiments with a single dose of hydrocortisone, thrombocytopenia was produced by a combination of irradiation and injection of anti-platelet antisera. Bleeding times were measured 30 min after the administration of anti-platelet serum in animals that had been irradiated 10 d previously.

All experiments were done with normal or thrombocytopenic rabbits in groups of eight. The animals were randomly treated with placebo or hydrocortisone and the bleeding time was measured by an observer without knowledge of the treatment category. Phase-contrast platelet counts were determined just before the bleeding times were done (7).

Experiments were designed to determine (a) the effects of a single dose of intravenous hydrocortisone and of daily intramuscular injections of hydrocortisone on both the jugular vein and microvascular bleeding times, and (b) the effect of topical hydrocortisone applied to the external surface of the vessel wall and of intraluminally administered hydrocortisone on the jugular vein bleeding time.

Single-dose intravenous hydrocortisone. Hydrocortisone was injected intravenously in a dosage range between 5 and 100 mg/kg into the marginal ear vein. Sodium chloride (0.9%) was used as a placebo in the control animals. The bleeding times were done 30 min after the administration of either hydrocortisone or placebo.

Daily intramuscular hydrocortisone. Animals were given 10 daily intramuscular injections of one of the following regimens: (a) hydrocortisone, 6 mg/kg daily for 10 d, (b) hydrocortisone, 3 mg/kg daily for 10 d, (c) sodium chloride (0.9%) for 5 d followed by hydrocortisone, 6 mg/kg for 5 d, (d) sodium chloride (0.9%) for 8 d followed by hydrocortisone, 6 mg/kg for 2 d, or (e) sodium chloride (0.9%) for 10 d.

Topical hydrocortisone. The effect of topical hydrocortisone was evaluated by applying cotton swabs saturated with concentrations of hydrocortisone ranging from 7.5 to 125 mg/ml to the jugular veins. Swabs that contained either topical hydrocortisone or sodium chloride (0.9%) (as placebo) were wrapped around the isolated jugular veins for 30 min, after which the jugular bleeding time was performed.

Intraluminal instillation of hydrocortisone. The jugular vein of the rabbit was isolated between ligatures and the circulation temporarily arrested. The blood was washed out of the isolated jugular vein segment through a cannulated tributary. By using this same tributary, 0.3 ml of hydrocortisone (100 mg/ml) or a placebo (0.9% NaCl) was instilled intraluminally. After a 30-min incubation period circulation was restored and after 5 min a bleeding time determination was done.

Effect of other prostaglandin synthesis inhibitors on the jugular bleeding time

Two other prostaglandin inhibitors, indomethacin and tranilcypromine, were instilled intraluminally into the jugular vein to test their effect on the jugular vein bleeding time and performed as described above.

¹Abbreviations used in this paper: 6-keto-PGF₁α, 6-keto-prostaglandin F₁α; PGI₂, prostacyclin.

Effect of local infusion of arachidonic acid, linoleic acid, or PGI₂ on jugular bleeding time

The jugular veins of the rabbits were isolated between ligatures and 0.3 ml of either hydrocortisone (100 mg/ml), indomethacin (0.1, 1.0, or 15 mg/ml in 100 mM sodium carbonate) (8), tranlylcypromine (0.1, 0.5, or 5 mg/ml), or isotonic diluent as placebo instilled intraluminally. After 30 min, the circulation was restored and the bleeding time was measured while either arachidonic acid (300 µg/ml), linoleic acid (300 µg/ml), PGI₂ (300 µg/ml in 0.05M Tris buffer, pH 8.9), or placebo were infused at a rate of 0.06 ml/min. In this way, the vessel wall and the puncture site were exposed to arachidonic acid, linoleic acid, or PGI₂ over the whole period that the bleeding time was being recorded. Comparisons were thus possible between one side treated with either arachidonic acid or PGI₂ and the other with either linoleic acid or a placebo.

Quantitation of flow rate from the puncture site in the jugular vein

The jugular veins of normal rabbits were isolated, flushed through with 0.9% saline, and one tributary was cannulated and connected to a saline reservoir so that it was subjected to a constant head of pressure. Hydrocortisone, indomethacin, tranlylcypromine, or a placebo were instilled intraluminally and removed after 30 min. The proximal ligature was kept in place and the vessel was punctured as described above. The fluid that escaped from the puncture site under this constant pressure head was collected for 10 min and the flow rate was determined. The experiments were repeated while arachidonic acid, (300 µg/ml), linoleic acid (300 µg/ml), PGI₂ (300 µg/ml), or a placebo were infused through a second cannulated tributary at a flow rate of 0.06 ml/min.

Measurement of PGI₂-like material extracted from the vessel wall

Jugular veins from normal and thrombocytopenic rabbits were assayed for PGI₂-like material 30 min after the intravenous infusion of hydrocortisone or the placebo with a [¹⁴C]-serotonin-release assay. Suspensions of washed rabbit platelets were prepared by the method of Ardlie et al. (6). 3 µCi of [¹⁴C]serotonin were added to the first washing fluid and incubated for 20 min at room temperature. After two washes, the platelets were suspended in Tyrode's solution that contained 0.35% bovine albumin and the platelet count was adjusted to 700,000/µl. The jugular veins were isolated and 4-cm lengths were removed, opened longitudinally, and washed with 0.05 M Tris buffer, pH 7.6 (9). The washed veins, which weighed ≈80 mg, were cut into 2-mm strips and placed in 1 ml of Tris buffer at 4°C. After stirring for 2 min, the supernate was obtained by centrifugation for 15 s at 12,000 g and this was tested for its inhibitory effect on [¹⁴C]serotonin release from rabbit platelets. This was done by incubating 0.1 ml of supernate from strips of jugular vein tissue or buffer with 1 ml of platelet suspension for 4 min at room temperature. Thrombin (0.1 ml), in a final concentration ranging from 0.1 to 0.01 U/ml, was added and after 7 min of incubation at room temperature, 0.1 ml of EDTA (1 dM) was added. The platelets were isolated by centrifugation for 1 min at 12,000 g and the amount of [¹⁴C]serotonin in the supernate was determined by liquid scintillation counting. Released radioactivity was calculated as a percentage of the total radioactivity in the platelets. The released radioactivity obtained when

platelets were incubated with supernate from the jugular vein was compared with the results obtained when platelets were incubated with buffer control.

Characterization of the inhibitor of platelet [¹⁴C]serotonin release obtained from jugular veins

The stability of the inhibitory activity of the supernate on [¹⁴C]serotonin release was studied by incubating the supernate at 37°C, 20°C, or 4°C, in Tris buffer adjusted to pH of 8.6 or 7.4, or by boiling the supernate for 15 s. The inhibitory activity was extracted from the supernate with cold diethyl ether as described by Weksler et al. (10). The effect of tranlylcypromine (500 µg/ml) was studied by adding it to the buffer in which the strips of jugular vein were incubated (9).

Measurement by radioimmunoassay of 6-keto-prostaglandin F_{1α} extracted from jugular vein wall

Antibody to 6-keto prostaglandin F_{1α} (6-keto-PGF_{1α}) was generously supplied by Dr. Bryan Smith. [³H]6-keto-PGF_{1α} was dissolved in 10 mM Tris-HCl buffer, pH 7.4; such that 0.1 ml contained 3,000 dpm. The quantity of antiserum which bound 50–80% of the radioactivity was then determined. A standard curve was established with concentrations of unlabeled 6-keto-PGF_{1α} ranging from 0.3 to 30 pmol. To the known standard was added 100 µl of [³H]6-keto-PGF_{1α}, then 40 µl of gammaglobulin (10 mg/ml) and 10 µl of antiserum to 6-keto-PGF_{1α} were added and incubated for 60 min at 37°C. Bound radioactivity was separated from unbound radioactivity by the addition of 250 µl of saturated cold ammonium sulphate. The supernate was prepared by centrifugation at 15,000 g for 2 min, then a 250-µl aliquot of supernate was transferred to a counting vial for scintillation counting. Supernates from jugular vein segments were handled in an identical fashion and a standard curve was run simultaneously with each assay.

Coagulation and platelet function tests

Blood was drawn from the rabbits before and 30 min after the intravenous infusion of hydrocortisone (100 mg/kg) and the following tests of blood coagulation and platelet aggregation were done: prothrombin time, activated partial thromboplastin time, whole blood clotting time, thrombin clotting time, euglobulin lysis time, fibrin plate lysis, and plasma fibrinogen (11). Platelet aggregation was performed by adding the following aggregating agents to platelet-rich plasma; ADP (1 µM), thrombin (0.1 U/ml), ristocetin (1.5 mg/ml), and arachidonic acid (1 mM). All concentrations are given as final concentrations.

Materials

Hydrocortisone as hydrocortisone sodium succinate, PGI₂ as the sodium salt, and 6-keto-PGF_{1α} were generously supplied by Upjohn Company of Canada, Don Mills, Ontario. [³H]6-keto-PGF_{1α} (10.9 Ci/mmol sp act) and rabbit antiserum to 6-keto-PGF_{1α} were the generous gifts of Dr. Bryan Smith, Jefferson Medical College, Philadelphia, Pennsylvania. Tranlylcypromine sulphate was generously supplied by Smith Kline & French Canada Ltd., Montreal, Quebec. Indomethacin (1-[*p*-chlorobenzoyl]-5-methoxy-2-methylindole-3-acetic acid), arachidonic acid (5,8,11,14-eicosatetraenoic acid) 99%

purity, linoleic acid (*cis*-9,*cis*-12-octadecadienoic acid), and ADP were obtained from Sigma Chemical Co., St. Louis, Mo. and ristocetin from H. Lundbeck & Co., Copenhagen. [¹⁴C]serotonin (5'-hydroxy-tryptamine creatinine sulphate), 58 mCi/mmol sp act, was obtained from Amersham Corp., Arlington Heights, Ill.

RESULTS

Effect of thrombocytopenia on the jugular vein and the microvascular bleeding times. The jugular bleeding time in 40 normal rabbits with a mean platelet count of $414 \pm 82.6 \times 10^3/\mu\text{l}$ was 89.6 ± 35.3 s. The relationship between the platelet count and the jugular bleeding time in animals made thrombocytopenic by either X-irradiation or heterologous platelet antiserum and in control animals is shown in Fig. 1. There was an inverse correlation between the bleeding time and the log of the platelet count ($r^2 = 0.89$, $P < 0.001$). The relationship between the bleeding time and platelet count when thrombocytopenia was produced by either irradiation or with heterologous platelet antiserum was similar. With parallel regression analysis there was no difference between the two sets of data ($P > 0.05$).

The microvascular bleeding time in 22 normal rabbits, with a mean platelet count of $350.7 \pm 80.5/\mu\text{l}$, was 76.7 ± 14.2 s. There was an inverse correlation between the microvascular bleeding time and the log of the platelet count ($r^2 = 0.86$, $P < 0.001$).

The effect of hydrocortisone on the jugular vein and microvascular bleeding times in normal and throm-

bocytopenic animals. In normal nonthrombocytopenic animals the intravenous injection of 100 mg/kg of hydrocortisone shortened both the jugular and microvascular bleeding times. In 18 normal rabbits, with a mean platelet count (± 1 SD) of $424.8 \pm 107.4 \times 10^3/\mu\text{l}$, the mean jugular bleeding time was 89.4 ± 34.3 s. After the intravenous infusion of 100 mg/kg of hydrocortisone the mean jugular bleeding time was reduced to 49.7 ± 29.3 s, a statistically significant difference ($P < 0.05$). Hydrocortisone, 100 mg/kg, significantly reduced the microvascular bleeding time in eight normal rabbits (platelet count $294.6 \pm 45.4 \times 10^3/\mu\text{l}$), from 83.3 ± 13.4 to 70.4 ± 12.3 s ($P < 0.05$).

A dose-response relationship was seen with both the jugular vein bleeding time and microvascular bleeding time when a single dose of hydrocortisone was injected intravenously in thrombocytopenic rabbits (Table I). Hydrocortisone, in doses of 25, 50, and 100 mg/kg, significantly reduced the jugular vein bleeding time ($P < 0.01$). The shortening of the jugular vein bleeding time by 100 mg/kg of a single infusion of intravenous hydrocortisone in rabbits made thrombocytopenic either by irradiation or by using heterologous platelet antiserum was similar and not statistically different. Hydrocortisone, in doses between 10 and 100 mg/kg, shortened the microvascular bleeding time, but no effect was noted at a dose of 5 mg/kg (Table I). The effect on the microvascular bleeding time of intravenous hydrocortisone in a dose of 100 mg/kg was most marked with platelet counts above $40,000/\mu\text{l}$, but a significant reduction was also observed when the platelet counts were between 1,000 and $10,000/\mu\text{l}$ (Table II).

The effect of daily administration of hydrocortisone by intramuscular injection in smaller doses is shown in Table III. At doses of 3 or 6 mg/kg administered for 10 d there was a significant reduction of the jugular bleeding time in thrombocytopenic animals. This effect of 6 mg/kg of hydrocortisone was less marked when administered for 5 d or less ($P < 0.01$). A similar effect was attained with 6 mg/kg given for 10 d to thrombocytopenic animals with the microvascular bleeding time (Table III).

The effect of the topical application of hydrocortisone is shown in Table IV. There was a significant shortening of the bleeding time at all concentrations tested (7.5–125 mg/ml) ($P < 0.01$). Multiple comparisons showed a significant difference ($P < 0.05$) among all dosage groups at concentrations of 125 and 60 mg/ml, which were not significantly different from each other.

Mechanism of the hydrocortisone effect. The possibility that hydrocortisone exerted its effect on the bleeding time by influencing prostaglandin synthesis by the vessel wall was explored by (a) studying the effects of two other inhibitors of prostaglandin synthesis (indomethacin and tranlylcypromine) on the jugular

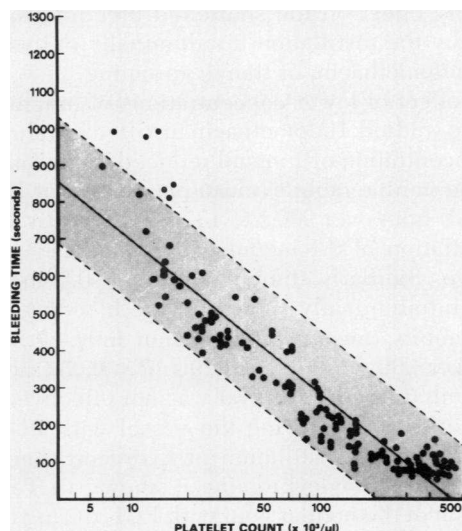


FIGURE 1 The relationship between the jugular bleeding time and the platelet count in thrombocytopenic rabbits. The thrombocytopenia was produced by either irradiation or heterologous platelet antiserum. This relationship may be expressed as $y = 1103.2 - 407.2 \log_{10} x$, where y is the bleeding time and x the platelet count ($r^2 = 0.89$, $P < 0.001$). The shaded area represents the 95% confidence limits.

TABLE I
The Effect of a Single Intravenous Dose of Hydrocortisone on the Jugular and Microvascular Bleeding Times

Bleeding time technique	Agent	Dose	No. of experiments	Platelet count ± 1 SD	Bleeding time ± 1 SD
		mg/kg		× 10 ³ /μl	s
Jugular	Placebo	—	51	20.2 ± 8.2	572.6 ± 110.8
	Hydrocortisone	25	18	12.7 ± 3.1	435.6 ± 122.8
	Hydrocortisone	50	16	21.6 ± 10.3	231.3 ± 46.8
	Hydrocortisone	100	40	25.5 ± 11.7	113.9 ± 44.5
Microvascular	Placebo	—	8	21.1 ± 7.4	870.9 ± 82.4*
	Hydrocortisone	5	8	28.9 ± 18.4	868.1 ± 90.2*
	Hydrocortisone	10	7	14.7 ± 6.4	492.0 ± 110.2
	Hydrocortisone	20	8	24.5 ± 15.4	391.8 ± 65.6
	Hydrocortisone	25	8	20.4 ± 7.7	272.9 ± 138.3
	Hydrocortisone	50	8	19.5 ± 8.3	263.4 ± 116.6
	Hydrocortisone	100	8	20.1 ± 12.7	222.3 ± 139.0

Analysis of variance indicated a significant difference ($P < 0.01$) in the mean jugular bleeding times among the different groups of animals given hydrocortisone or placebo. With the microvascular bleeding time there was no significant difference in the bleeding time among the three groups of animals receiving 25, 50, and 100 mg/kg of hydrocortisone, but there was a significant ($P < 0.01$) reduction of each of these dosage groups compared to the placebo group using the rank sum test. (12)

* Seven of eight determinations resulted in bleeding times >900 s.

vein bleeding time and (b) by investigating the effects of the local infusion of arachidonic acid, linoleic acid, and PGI₂ on the bleeding time of rabbits that had been exposed to hydrocortisone, indomethacin, or tranilcypromine. The effect of the intraluminal instillation of hydrocortisone, indomethacin, or tranilcypromine in the presence or absence of infused arachidonic acid is shown in Table V. Perfusion of the punctured jugular vein segment in thrombocytopenic animals with arachidonic acid alone but no effect on the prolonged bleeding time. The intraluminal instillation of hydrocortisone markedly shortened the bleeding time ($P < 0.01$), but this effect was reversed by infusion of arachidonic acid. The intraluminal instilla-

tion of indomethacin or tranilcypromine also significantly reduced the bleeding time but these effects were not reversed by perfusing the vessel segment with arachidonic acid. The perfusion of linoleic acid at the same concentration as arachidonic acid did not have any effect on the shortened bleeding time produced by the instillation intraluminally of hydrocortisone, indomethacin, or tranilcypromine.

The effect of lower concentrations of indomethacin was also studied. Indomethacin instilled intraluminally at a concentration of 1 mg/ml reduced the mean bleeding time in nine rabbits (mean platelet count $12.8 \pm 3.8 \times 10^3/\mu\text{l}$) from over 900 s to 137.6 ± 41.5 s ($P < 0.05$). A concentration of 0.1 mg/ml of indomethacin was not effective. Similarly, tranilcypromine, 0.5 mg/ml instilled intraluminally shortened the bleeding time in eight rabbits (mean platelet count $9.9 \pm 4.7 \times 10^3/\mu\text{l}$) from over 900 s to 162.5 ± 37.9 s ($P < 0.05$), however, a concentration of 0.1 mg/ml was not effective.

The effect of perfusing the vessel with PGI₂ after the intraluminal instillation of hydrocortisone, indomethacin, or tranilcypromine is shown in Table VI. Perfusion of the jugular vein with PGI₂ during the performance of the bleeding time reversed the effects of hydrocortisone, indomethacin, and tranilcypromine. The perfusion of the jugular vein with linoleic acid on the opposite side had no effect.

Perfusion of the jugular vein with PGI₂ also reversed the effect of a single intravenous dose of hydrocorti-

TABLE II
The Effect of a Single Intravenous Dose of Hydrocortisone (100 mg/kg) on the Microvascular Bleeding Time in Relationship to the Platelet Count

Platelet count	No. of experiments	Mean platelet count ± 1 SD	Mean bleeding time ± 1 SD
× 10 ³ /μl		× 10 ³ /μl	s
1–10	26	2.9 ± 1.0	317.8 ± 138.1
11–40	18	18.8 ± 4.0	183.9 ± 98.0
41–100	14	72.4 ± 19.9	98.7 ± 32.6

Analysis of variance showed a significant difference ($P < 0.05$) among the three groups.

TABLE III

The Hemostatic Effect of Daily Intramuscular Hydrocortisone on the Jugular and Microvascular Bleeding Times in Thrombocytopenic Rabbits (Platelet Count $< 20 \times 10^3/\mu\text{l}$)

Agent	Dose	Duration	No. of experiments	Jugular bleeding time ± 1 SD	No. of experiments	Microvascular bleeding time ± 1 SD
	mg/kg	d		s		s
Placebo	—	10	25	872.0 \pm 70.3	8	>900*
Hydrocortisone	3	10	12	288.8 \pm 125.7	—	ND
Hydrocortisone	6	10	30	254.1 \pm 76.8	8	379.3 \pm 134.1
Hydrocortisone	6	5	13	316.5 \pm 55.7	6	550.3 \pm 138.6
Hydrocortisone	6	2	17	554.5 \pm 34.4	—	ND

Analysis of variance showed significant differences ($P < 0.01$) among groups of animals given 6 mg/kg of hydrocortisone or placebo for different periods. There was no significant difference between the mean bleeding times of animals given 3 and 6 mg/kg of hydrocortisone for 10 d on the jugular bleeding time. (ND, not determined)

* All eight determinations resulted in bleeding times >900 s.

sone. Eight rabbits (mean platelet count of $16.3 \pm 5.1 \times 10^3/\mu\text{l}$) treated with 100 mg/kg of hydrocortisone had a mean jugular bleeding time of 106.9 ± 53.2 s on the side perfused with linoleic acid, and the side perfused with PGI_2 had a mean jugular bleeding time of 868.6 ± 61.3 s ($P < 0.05$).

The effect of the various agents on the size of the puncture wound was assessed by measuring the rate of flow of saline from a standard puncture wound in isolated perfused jugular vein segments exposed to a constant head of pressure (Table VII). Hydrocortisone, indomethacin, and tranlycypromine instilled intraluminally all reduced the rate of flow of saline from a standard puncture wound compared to a placebo control ($P < 0.01$). The perfusion of arachidonic acid during the experiment reversed the effect of hydrocortisone, but not the effect of indomethacin and tranlycypromine. PGI_2 reversed the reduced flow rate produced by all three agents, however, linoleic acid had no effect.

Intravenous hydrocortisone in doses of 100 or 50 mg/kg also significantly reduced the flow rate compared to placebo ($P < 0.05$). This effect was also reversed by perfusion of the vein segment by arachidonic acid and PGI_2 , but not by linoleic acid.

Effect of hydrocortisone on the generation of PGI_2 -like material and 6-keto- $\text{PGF}_{1\alpha}$. The supernates from jugular veins from normal rabbits treated either with hydrocortisone or placebo were tested for their ability to inhibit the release of [^{14}C]serotonin from thrombin treated rabbit platelets. Vessel wall supernate inhibited thrombin-induced [^{14}C]serotonin release at final thrombin concentrations of 0.01, 0.02, and 0.025 U/ml (Table VIII). This inhibitory effect was significantly less with the supernates from rabbits treated with hydrocortisone. A similar reduction in the concentration of PGI_2 -

like material was obtained from the supernatant fluids of jugular veins of thrombocytopenic rabbits treated with hydrocortisone (100 mg/kg) and of normal rabbits treated for 10 d with 6 mg/kg of hydrocortisone.

This inhibitory effect of the jugular vein supernates on thrombin-induced [^{14}C]serotonin release was abolished by boiling the supernate at 100°C for 15 s or by incubating it at 20°C or 37°C for 60 min at a pH of 7.4. However, most of the activity remained when it was incubated at a pH of 8.6 for 24 h at 4°C . The inhibitory activity was extractable in ether but was not present in the supernate from jugular vein strips that had been incubated with tranlycypromine (500 $\mu\text{g}/\text{ml}$) (9).

Measurement by radioimmunoassay of 6-keto- $\text{PGF}_{1\alpha}$ extracted from the jugular vein walls revealed significantly lower levels in those animals that had been pretreated with hydrocortisone compared to those that

TABLE IV

The Effect of Topical Hydrocortisone on the Jugular Bleeding Time in Thrombocytopenic Rabbits

Agent	Dose	No. of experiments	Platelet count ± 1 SD	Bleeding time ± 1 SD
	mg/ml		$\times 10^3/\mu\text{l}$	s
Placebo	—	20	12.2 \pm 2.9	>900*
Hydrocortisone	7.5	7	10.2 \pm 2.3	528.0 \pm 48.4
Hydrocortisone	15	11	11.6 \pm 3.9	319.5 \pm 27.2
Hydrocortisone	30	12	10.0 \pm 3.9	146.8 \pm 49.3
Hydrocortisone	60	12	7.5 \pm 3.8	104.3 \pm 35.5
Hydrocortisone	125	18	10.6 \pm 4.1	100.2 \pm 36.5

Multiple comparisons showed a significant difference ($P < 0.05$) among all the dosage groups and placebo except for the groups given 125 and 60 mg/ml, which were not significantly different from each other.

* All 20 determinations resulted in a bleeding time >900 s.

TABLE V
The Effect on the Jugular Bleeding Time of the Intraluminal Instillation of Hydrocortisone (100 mg/ml), Indomethacin (15 mg/ml), or Tranylcypromine (5 mg/ml), Followed by the Intraluminal Perfusion of Arachidonic Acid or a Placebo in Thrombocytopenic Rabbits (Platelet Count < 30 × 10³/μl)

Agent instilled intraluminally	Agent perfused intraluminally	No. of experiments	Bleeding time ± 1 SD	P value†
<i>s</i>				
Placebo	Placebo	9	>900*	NS
Placebo	Arachidonic acid	12	>900*	
Hydrocortisone	Placebo	17	92.1 ± 25.5	<0.01
Hydrocortisone	Arachidonic acid	14	>900*	
Indomethacin	Placebo	18	115.5 ± 33.2	NS
Indomethacin	Arachidonic acid	12	100.6 ± 44.7	
Tranylcypromine	Placebo	11	263.1 ± 162.5	NS
Tranylcypromine	Arachidonic acid	12	214.8 ± 108.6	

* All determinations resulted in a bleeding time >900 s.

† Comparison of groups by *t* test or rank sum test (12) (NS, not significant at the 0.05 level).

had been given a placebo. The supernatant fluid of jugular veins from eight placebo-treated animals had a mean concentration of 19.5 pmol/100 μl of 6-keto-PGF₁α, whereas the supernatant fluid from jugular veins from eight animals pretreated with 100 mg/kg of intravenous hydrocortisone had a mean concentration of 0.6 pmol/100 μl, a statistically significant difference, (*P* < 0.05). Similarly, there was a reduction of 6-keto-PGF₁α in the supernatant fluid from the jugular veins of six rabbits treated for 10 d with 6 mg/kg of hydrocortisone. The mean level was 5.1 pmol/100 μl compared to a mean concentration of 16.0 pmol/100 μl in control rabbits treated with a placebo for 10 d (*P* < 0.05).

Effect of hydrocortisone on coagulation and platelet function tests. The intravenous infusion of 100 mg/kg of hydrocortisone did not alter the results of the thrombin clotting time, prothrombin time, partial thromboplastin time, whole blood clotting time, euglobulin lysis time, fibrin plate lysis, nor did it augment platelet aggregation with ADP, collagen, ristocetin, or arachidonic acid.

DISCUSSION

The results of these experiments demonstrate that hydrocortisone administered either systemically,

TABLE VI
The Effect of PGI₂ on the Jugular Bleeding Time Produced by the Intraluminal Instillation of Hydrocortisone (100 mg/ml), Indomethacin (15 mg/ml), or Tranylcypromine (5 mg/ml) in Thrombocytopenic Rabbits (Platelet Count < 30 × 10³/μl)

Agent instilled intraluminally	Agent perfused intraluminally	No. of experiments	Bleeding time ± 1 SD	P value*
<i>s</i>				
Hydrocortisone	Linoleic acid	8	106.9 ± 53.2	<0.05
Hydrocortisone	Prostacyclin	8	868.6 ± 61.3	
Indomethacin	Linoleic acid	9	83.1 ± 41.3	<0.05
Indomethacin	Prostacyclin	9	892.7 ± 22†	
Tranylcypromine	Linoleic acid	8	87.5 ± 32.2	<0.05
Tranylcypromine	Prostacyclin	8	>900§	

* Comparison of groups by sign test (13).

† Eight of nine determinations resulted in bleeding times >900 s.

§ All eight determinations resulted in bleeding times >900 s.

TABLE VII
The Effect of Intraluminal Instillation of Hydrocortisone, Indomethacin, or Tranilcypromine on the Flow Rate from a Puncture Wound in the Jugular Vein, and the Effect of Arachidonic Acid, PGI₂, or Linoleic Acid as a Control

Agent	Intraluminal perfusing agent	No. of experiments	Flow rate \pm 1 SD ml/min	P value*
Hydrocortisone 100 mg/ml	Linoleic acid	9	1.00 \pm 0.13	<0.01
	Arachidonic acid	9	2.22 \pm 0.15	
	Linoleic acid	10	0.67 \pm 0.06	<0.01
	Prostacyclin	10	1.75 \pm 0.09	
Indomethacin 15 mg/ml	Linoleic acid	8	0.98 \pm 0.12	NS
	Arachidonic acid	8	0.94 \pm 0.14	
	Linoleic acid	8	1.13 \pm 0.17	<0.05
	Prostacyclin	8	1.81 \pm 0.08	
Tranilcypromine 5 mg/ml	Linoleic acid	8	0.56 \pm 0.05	NS
	Arachidonic acid	8	0.56 \pm 0.07	
	Linoleic acid	6	0.65 \pm 0.05	<0.05
	Prostacyclin	6	1.77 \pm 0.08	
Placebo	Linoleic acid	12	1.96 \pm 0.17	NS
	Saline	12	1.93 \pm 0.15	

* Comparison of paired groups of results by paired *t* test (NS, not significant at the 0.05 level).

topically, or intraluminally, reduces the jugular vein bleeding time and that systemic hydrocortisone reduces the microvascular bleeding time in thrombocytopenic animals. A dose-response relationship was obtained with both bleeding methods, but the microvascular bleeding time appeared to be more sensitive to lower doses of hydrocortisone. This effect could be caused by differences in the degree of inhibition of PGI₂ synthesis by hydrocortisone or it could reflect a difference in the mechanism of hemostasis in these two circulations.

Systemic hydrocortisone also shortens both bleeding times in nonthrombocytopenic animals. This is in con-

trast to the report by Thong et al. (14) who did not demonstrate a significant effect on the bleeding time of 80 mg of prednisone given orally for 2 d to normal human volunteers. This apparent discrepancy could be related to the different doses of corticosteroids used or to the differences in the duration of their administration.

The effect of hydrocortisone could not be attributed to alterations in blood coagulation, fibrinolysis, or platelet reactivity (15). The effect of hydrocortisone was dose related and was more marked when the drug was administered for 10 d than for 2 or 5 d. The shortest bleeding times were obtained when the platelet count

TABLE VIII
Thrombin-Induced Platelet [¹⁴C]Serotonin Release Produced by the Supernates of Jugular Veins from Rabbits Treated with a Single Dose of Intravenous Hydrocortisone (100 mg/kg) or Placebo

Rabbits treated with	No. of experiments	[¹⁴ C]serotonin release by thrombin		
		0.025 U/ml	0.02 U/ml	0.01 U/ml
		%	%	%
Hydrocortisone	6	52.0 \pm 3.7	44.3 \pm 8.3	24.7 \pm 2.0
Placebo	6	20.7 \pm 10.4	12.3 \pm 8.8	3.2 \pm 0.8
		P* < 0.01	P < 0.01	P < 0.01

* Statistical significance was calculated by analysis of variance.

was greater than 10,000/ μ l but the bleeding time was also shortened when the platelet count was below 10,000/ μ l.

Kitchens (16) has recently reported that treatment of rabbits with prednisone for 5 d protects the endothelium from the thinning and fenestrations induced by thrombocytopenia. In addition, mean vessel wall thickness was increased in the prednisone treated rabbits compared with control. Maca et al. (17) reported changes in endothelial cell morphology related to increased protein synthesis caused by incubation with glucocorticoids for 24–48 h.

These observations could, in part, explain our findings with the smaller doses of hydrocortisone that were found to be most effective when given for 10 d but would not explain the effects of large doses of hydrocortisone on the bleeding time that were observed within 30 min of administration. Furthermore, we demonstrated that even at these lower doses hydrocortisone reduced PGI₂ production by the vessel wall when given for a period of 10 d.

Hydrocortisone has been reported to inhibit prostaglandin synthesis by intact cells and it has been postulated that this effect is caused by inhibition of phospholipase A₂ that catalyzes the hydrolysis of arachidonic acid from membrane phospholipid (5, 18–23). In contrast, hydrocortisone has been reported to have no effect on prostaglandin synthesis in platelets (24). Corticosteroids have also been shown to inhibit the vasodilation that occurs in association with synthesis of prostaglandins by the vessel wall (25, 26). Vessel injury is normally associated with transient vasoconstriction which is followed by vessel relaxation. We suggest that the vessel relaxation after constriction is contributed to by smooth muscle-relaxing prostaglandins that are released from the vessel wall in response to injury (27–29), and that the inhibition of the synthesis of these prostaglandins leads to a sustained vasoconstriction. This vasoconstriction has a hemostatic effect when the vessel wall in either normal or thrombocytopenic animals is damaged, or the vessels in the microcirculation transected. This hypothesis is supported by a number of indirect and direct observations. Indomethacin, an inhibitor of prostaglandin synthesis and tranlycypromine, an inhibitor of PGI₂ synthesis (9, 10) also shortened the bleeding time in thrombocytopenic animals, but unlike hydrocortisone, the effects of these two agents on the bleeding time was not reversed by vessel perfusion with arachidonic acid. These findings are consistent with the view that hydrocortisone inhibits prostaglandin synthesis by preventing the hydrolysis of arachidonic acid from membrane phospholipid and that the other two inhibitors of prostaglandin synthesis act further down in the pathway of prostaglandin synthesis.

Infusion of PGI₂ into the jugular vein while the bleeding time was being performed reversed the effects of hydrocortisone, indomethacin, and tranlycypromine on the jugular bleeding time. These observations are also consistent with the hypothesis that these agents shorten the bleeding time by inhibiting the generation of endogenous prostacyclin by the vessel wall.

Indirect evidence that these agents produce sustained vasoconstriction was obtained by measuring the rate of fluid loss from punctured jugular vein segments that were perfused under a constant pressure head. All three agents tested reduced the rate of fluid loss and, as with the bleeding time, this effect was reversed by arachidonic acid in the hydrocortisone treated animals, but not in the animals treated with indomethacin or tranlycypromine. Again, prostacyclin reversed the effect in the animals treated with either hydrocortisone, indomethacin or tranlycypromine. Finally, it was demonstrated that in the doses used, hydrocortisone inhibited the formation by the vein wall of a substance with PGI₂-like activity. This substance was extractable by ether, was unstable when boiled for 15 s or when incubated for 1 h at 20°C or 37°C at a pH of 7.4, but was more stable when incubated at a pH of 8.6. PGI₂-like material was not extractable from jugular veins if the vessels had been incubated with tranlycypromine. In addition, levels of 6-keto-PGF₁ α extracted from the jugular vein walls were significantly lower in the veins of animals pretreated with hydrocortisone than in control animals. Because PGI₂ is a precursor of 6-keto-PGF₁ α , it seems reasonable to conclude that PGI₂ synthesis was reduced in the vessels from animals treated with hydrocortisone (30). This is consistent with the recent observations that betamethasone blocks PGI₂ synthesis in cultured endothelial cells (31).

The results of our experiments are therefore consistent with the hypothesis that the effect of hydrocortisone on the bleeding time in thrombocytopenic animals is related, at least in part, to inhibition of synthesis of a smooth muscle-relaxing prostaglandin (or prostaglandins), which has the characteristics of PGI₂, and that hydrocortisone shortens the bleeding time in thrombocytopenic animals by having a direct effect on the vessel wall.

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