Susceptibility to Thrombosis in Normal Young, Aging, Cortisone-treated, Heparinized and X-irradiated Hamsters as Tested by Topical Application of Thrombin

By Herbert J. Berman, George P. Fulton, Brenton R. Lutz and David L. Pierce

Since thromboembolism is a common cause of death, predisposing factors are important and reliable tests for susceptibility are valuable. Several in vitro methods have been used for determining the agglutinability and adhesiveness of blood platelets. Recently a method has been described for early detection of thrombosis, based on the pain resulting from inflation of a pneumatic cuff applied to the calf or thigh. Furthermore, altered platelet counts have been associated with thrombotic episodes. However, we have found no references in the literature to in vivo methods for measuring actual thrombus susceptibility in man or experimental animals.

A possible test procedure for thrombus susceptibility in the hamster was suggested by the observation that thrombin applied topically to the everted cheek pouch produces platelet thromboembolism, varying in degree according to the condition of the animal. Low concentrations of thrombin produced thrombi in aging hamsters and during cortisone administration, whereas higher concentrations were required to produce thrombi following treatment with heparin and during thrombocytopenia induced by total body x-irradiation. In this paper we describe an in vivo test for measuring susceptibility to platelet thrombosis in the hamster, and report some of the findings.

Methods

Young adult golden hamsters (Mesocricetus auratus), 9 to 15 weeks old, were used, except for the observations on aging hamsters (at least one year old). Both males and females were included in all experiments and no obvious differences in thromboembolic tendency were associated with the sex of the animal.

The test for thrombus susceptibility was made on the everted cheek pouch of the hamster anesthetized with Nembutal (pentobarbital sodium, Abbott) given by intraperitoneal injection of 9 mg./100 Gm. of body weight with 2 mg. supplements as needed. The cheek pouch was prepared for transillumination by pinning over a transparent optical block. The blood vessels were exposed for topical application of thrombin by removal of a portion (3 mm. in diameter) of the upper wall of the pouch and underlying loose connective tissue. The approximate threshold concentration of thrombin required to produce intravascular platelet thrombi was determined by flooding the preparation with increasingly concentrated solutions of thrombin until platelet thrombi were obtained. A standard thrombin solution was prepared by adding 1 cc. of distilled water to a vial containing 1000 X.I.H.

From the Department of Biology, Boston University, Boston, Mass.

Supported by grant No. H-902 (c3) from the National Heart Institute, National Institutes of Health, P.H.S. and Atomic Energy Commission Contract No. (301)-807.

The authors acknowledge the statistical assistance of Professor Elmer B. Mode, Chairman of the Mathematics Department, Boston University, and the photographic assistance of Frederick W. Maynard.

Submitted December 6, 1954; accepted for publication January 27, 1955.
units of powdered thrombin (Thrombin, Topical, bovine origin, Parke, Davis & Company). The test solutions were made by dilution of the standard with mammalian Ringer's solution. Six concentrations (expressed as per cent of the standard solution) were used: 1.25, 2.5, 5.0, 10.0, 50.0 and 100.0 per cent, with respective N.I.H. unit values/cc. of 12.5, 25, 50, 100, 500 and 1000. In conducting the test, the 1.25 per cent thrombin solution was applied to the cheek pouch for 15 minutes and then replaced with mammalian Ringer's solution for 15 minutes prior to application of the second concentration of thrombin. The procedure was continued until two or more platelet thrombi were detected by microscopy at 90 or 150 magnifications. The effective thrombin solution was considered to be the approximate threshold concentration.

The thrombin test has been used for determining the susceptibility to thrombosis of untreated normal hamsters (control group) and irradiated, cortisone-treated, heparinized and aging hamsters. All results were analyzed statistically by means of a 2 by 2 comparative trial test.¹² ¹³

**RESULTS**

Thrombin applied topically to the everted cheek pouch of the hamster produced white thrombi (composed of platelets) in uninjured blood vessels containing circulating blood (fig. 1B, C and D). Red thrombi were formed only in stagnant blood resulting from occluding platelet thrombi. An increased number of leukocytes adhered to the venular endothelium. Thrombus formation occurred in the venules for the most part and seldom in arterioles or capillaries. Larger doses of thrombin produced thrombi in arterioles (fig. 1E), although the venules were involved first. Consequently, the most vulnerable part of the circulatory system for thrombosis was the venous portion.

**Thrombus Susceptibility in Normal Hamsters**

In the control group of 27 normal young hamsters (9 to 15 weeks old), the approximate threshold concentration of thrombin required to produce platelet thrombi within 15 minutes was 2.5 and 5.0 per cent (25 and 50 N.I.H. units/cc.). A total of 10 hamsters (37 per cent) developed thrombi with a 2.5 per cent concentration and the remaining 17 (63 per cent) required a 5.0 per cent solution.

**Effect of Cortisone Treatment**

The effect of cortisone acetate (Cortone, Merck) on thrombus susceptibility in the hamster has been tested during treatment with three different subcutaneous doses (10 mg. daily for 11 days, 5 mg. daily for 17 days and 5 mg. on alternate days for 2 weeks). The findings in all three groups were consistent. Seventy-four per cent of the cortisone-treated hamsters developed thrombi with a 1.25 per cent thrombin solution (figs. 2A and 4). By statistical methods, these values were shown to be significantly different from those obtained in normal untreated hamsters. Consequently, an increased susceptibility to thromboembolism is indicated during cortisone treatment.

Previous reports on the effect of cortisone on blood coagulation and thrombosis are controversial. Cosgriff and co-workers¹⁴ ¹⁵ found hypercoagulable blood in patients on cortisone therapy and later reported an increased incidence of thrombosis. These findings have been disputed,¹⁶ ¹⁷ and Russek, Zohman and Russek¹⁸ concluded that danger of thrombotic complications from the use of cortisone is not clinically significant. In the hamster, cortisone produces arterial constrictive...
Fig. 1.—Platelet thrombosis produced by topical thrombin applied to hamster cheek pouch. Thrombi are absent (A) prior to application of thrombin (100 X). In B and D, platelet thrombi are shown in venous vessels (v) at 15 minutes after 5 per cent thrombin (100 X, 260 X). No thrombi are found in the arterial vessels. In C, 100 per cent thrombin produces thrombi in arterioles (a) as well as venules.
CONC. (%)
THROMBIN

0.0 5.0 2.5 1.25

A

CONC. (%)
THROMBIN

10.0 5.0 2.5 1.25

B

CONC. (%)
THROMBIN

100.0 50.0 10.0

C

PERCENT SHOWING PLATELET THROMBI (15 minutes)

SUSCEPTIBILITY TO THROMBOSIS
tion, extensive sticking of leukocytes to the endothelium of venous vessels, decreased numbers of tissue mast cells, and a marked increase in the ease with which topical thrombin produces platelet thrombi. Cortisone, at least in the hamster, appears to predispose to platelet thrombosis.

Effect of Aging
In aging hamsters also, an increased susceptibility was suggested by the results of the thrombin test, agreeing with the well known clinical findings of a marked increment in thromboembolism in patients over 40. A total of 87 per cent developed thrombi with a 2.5 per cent thrombin solution as compared with only 37 per cent in the control group of young animals (figs. 2B and 4). Our in vivo results in the aging hamster are in accord with the in vitro findings of increased agglutinability of platelets in man with age.

Heparin
An increased resistance to thrombus formation, as measured by the thrombin test, was obtained following intravenous injection of Heparin Sodium (1000 U.S.P. units, Abbott). The results are shown in figures 2C and 4.

Although heparin is known to stop or prevent red thrombosis, the effect on platelet agglutination, as shown by the published literature, is far from clear. A much higher concentration of heparin is reported to be necessary for inhibition of platelet agglutination than for prevention of blood coagulation. However, increased platelet agglutination has been reported in vivo as a result of injections of sufficient heparin to extend the clotting time of blood appreciably. In vitro tests have yielded conflicting results such as a decreased adhesiveness of platelets and an increased adhesiveness. Moreover, Rovatti reported an accentuated agglutinability of platelets in vitro in the presence of decreased adhesiveness to the glass container. In the present experiments using relatively enormous doses of 1000 U.S.P. units of heparin/100 Gm. of body weight, the development and propagation of large platelet thrombi in response to topical thrombin was inhibited. Small circulating platelet emboli were observed in some cases, especially immediately after injection.

X-irradiation
The susceptibility to thrombus formation was decreased by total body x-irradiation (figs. 3B and 4) with an approximate LD50/30 dose of 880r, and with

---

Graph A shows the average platelet thrombus susceptibility in hamsters after daily treatment with 5 or 10 mg. of cortisone for approximately 2 weeks, as tested by topical thrombin. The solid bars represent the controls and the hatched bars represent cortisone-treated hamsters. The fraction to the right of each bar represents the ratio of the number of hamsters developing platelet thrombi to the total number used. Statistically significant increases in susceptibility are shown with 2.5 per cent thrombin (2 X 2 comparative trial, \( z = 4.50 \)) and 1.25 per cent. Graph B shows the average platelet thrombus susceptibility in aging hamsters (1 year or older). Statistically significant increases in susceptibility are shown with 2.5 per cent thrombin \( (z = 2.48) \). Graph C shows the average platelet thrombus susceptibility in hamsters receiving heparin (1000 U.S.P. units). Statistically significant decreases in susceptibility are shown with 2.5 per cent thrombin \( (z = 2.02) \).
SUSCEPTIBILITY TO THROMBOSIS

A

CONC. THROMBIN (%)

0 10 20 30 40 50 60 70 80 90 100

PERCENT SHOWING PLATELET THROMBI (10 minutes)

B

CONC. THROMBIN (%)

0 10 20 30 40 50 60 70 80 90 100

PERCENT SHOWING PLATELET THROMBI (10 minutes)

C

PLATELET COUNT (1000/µl)

0 50 100 150 200 250 300 350 400 450 500

DAYS AFTER X-IRRADIATION

13 Hamsters
BEm\m.X, mi'tLTox, LUTZ

CORTISONE  AGING  CONTROL  X-RAY  HEPARIN

Fig. 4.—Comparative effects of cortisone, aging, x-ray and heparin on platelet thrombus susceptibility, as tested by 2.5 per cent topical thrombin. The fraction to the right of each bar represents the ratio of the number of hamsters developing platelet thrombi to the total number used.

1500r, approximately 100 per cent lethal for the hamster within 7 to 8 days. Hematologic findings in the x-irradiated hamster have been described previously. No significant change occurred in thrombus susceptibility, as compared with the controls, during the first 4 to 5 days following irradiation (fig. 3A). The decreased susceptibility was demonstrated subsequently at 6 to 11 days and a correlation was found with the platelet count, determined by the method of Rees and Ecker (fig. 3C). With a thrombocytopenia of 50,000 platelets cu.mm. or lower, platelet thrombi were not produced by thrombin solutions, even by those of 100 per cent concentration. The blood usually continued to circulate, but red thrombi formed eventually. With platelet concentrations of 100,000/cu. mm. or more, the susceptibility to platelet thrombosis remained within the range established for the control hamsters.

Discussion

Although thrombin is generally believed to produce red thrombosis, white thromboembolism predominated in our experiments. Since the thrombin utilized

Fig. 3.—Effect of total body x-irradiation on platelet thrombus susceptibility.

Graph A shows the average platelet thrombus susceptibility during the first 60 per cent of the survival period (1st to 5th day) following total body x-irradiation with 1500 r, as tested by topical thrombin. The solid bars represent control hamsters and the hatched bars represent irradiated hamsters. The fraction to the right of each bar represents the ratio of the number of hamsters developing platelet thrombi to the total number used. Platelet thrombosis occurred in 100 per cent of both control and irradiated hamsters with 5 per cent thrombin. No statistically significant differences were found with 2.5 per cent thrombin (2 X 2 comparative trial, z = 0.28). Graph B shows the average platelet thrombus susceptibility during the final 40 per cent of the survival period (5th to 7th day) following total body x-irradiation with 1500 r. Statistically significant decreases in susceptibility are shown with 2.5 per cent thrombin (z = 1.82), 5.0 per cent (z = 5.08), 10 per cent, and 100 per cent. In graph C the results of the thrombus susceptibility test in 13 x-irradiated hamsters (840-900 r) are correlated with the platelet counts. Severely thrombocytopenic hamsters died from pericardial hemorrhage after cardiac puncture.
SUSCEPTIBILITY TO THROMBOSIS

(Parke, Davis) is an impure product, the component producing platelet thrombi is not known. For this reason, a purified preparation* was tested and found to be effective, but less potent than the commercial preparation. Further work is in progress and other coagulation factors are being investigated for thrombus potentiality.

The thrombus-forming potentiality of thrombin was originally tested in vivo because of its well known intermediate role in blood coagulation. Although the thrombin test appears to give an index of predisposition to thrombosis, the exact physiologic basis is not clear as yet. It seems unlikely that thrombin acts by injuring the vascular endothelium. The number of circulating blood platelets might provide an explanation, since the initial thrombus in circulating blood originates from platelets. The results of the thrombus susceptibility test (topical thrombin) on hamsters with irradiation-induced thrombocytopenia support this viewpoint. With decreased platelet concentrations of 50,000 cu.mm. or less, thrombin solutions even as high as 100 per cent did not produce platelet thrombi. However, when the platelet count exceeded 100,000 cu.mm., thrombi formed as readily as in the control group. Furthermore, the hemorrhagic manifestations produced by irradiation may be alleviated by elevating the platelet concentration of the blood by injecting platelet rich plasma. These facts suggest a relatively critical minimum platelet concentration required for platelet thromboembolism, at least in x-irradiated hamsters. On the other hand, the platelet concentration cannot account for the increased susceptibility to thromboembolism in cortisone-treated hamsters and the decreased susceptibility during treatment with heparin. In these circumstances we found no correlation with platelet counts.

Variation in the agglutinability of platelets may be a major factor and possible explanation for the utility of the thrombin susceptibility test. Stefanini and Silverberg reported that the capacity of blood to undergo platelet agglutination is related to the prothrombin activity. However, several factors may affect prothrombin consumption. As yet, the exact causative factor or combination of factors in the coagulation process predisposing to platelet agglutination has not been determined. The agglutinability of platelets may vary considerably under different conditions. For example, thrombin per se will agglutinate unwashed platelets but will not agglutinate well washed platelets. Nevertheless the ease with which thrombi form in vivo after topical application of thrombin appears to be an index of thrombus susceptibility.

SUMMARY

1. Thrombin applied topically to the everted cheek pouch of the hamster produced platelet and not red thrombi in exposed, uninjured blood vessels with circulating blood. Red thrombi were produced in stagnant blood. Thrombus formation occurred in the venules for the most part and seldom in arterioles or capillaries.

2. An in vivo test for platelet thrombus susceptibility, based on the thrombin reaction and the resistance of the hamster to thrombosis, has been described.

* Kindly supplied by Dr. Walter H. Seegers, Wayne University College of Medicine.
3. Thrombus susceptibility, measured by the thrombin test, increased with age and during cortisone treatment, and decreased after heparin injection and following large doses of whole body x-irradiation.

4. The thrombin susceptibility test could be correlated with the platelet count in x-irradiated hamsters, showing a relatively critical minimum concentration of blood platelets (100,000/cu.mm.) required for platelet thrombosis.

5. The relationship of platelet concentration to platelet thrombus formation and predisposition to hemorrhage has been discussed.

**SUMMARIO IN INTERLINGUA**

1. Le application topic de thrombina al evertite bursa vestibular de hamsters causava thrombos plachettal, sed nulle thrombos rubie, in exponite non-ledite vasos con sanguine circulante. In sanguine stagnante, del altere latere, thrombos rubie eseva formate. Le formation del thrombos occurreva principalmente in venulas, rarmente in arteriolas o capillares.

2. Es describite un proba in vivo del susceptibilitate de hamsters a thrombos plachettal. Ilo es basate super le reactiomi a thrombina e le resistentia del animales a thrombosis.

3. Le susceptibilitate a thrombos, mesurate per le reaction a thrombina, acerceseva con le etate del animales e durante tractamento a cortisona. Ilo de-cresceva post injectiones de heparina e post le application de grande doses de roentgeniorradiation total.

4. In hamsters roentgeniorradiate il eseva possibile correlationar le proba del susceptibilitate a thrombina con le contation de plachettas. Con concentrations plachettal reduce a infra 50.000 per mm², nulle thrombos plachettal resultava mesmo de solutiones de 100 pro cento de thrombina. Quando le concentrationes plachettal excedeva 100.000 per mm², thrombos se formava tanto promptemente in le animales irradiate como in le gruppo de controlo.

5. Es discutite le relation del concentration plachettal al formation de thrombos plachettal e al predisposition a hemorrhagias.

**REFERENCES**

840  SUSCEPTIBILITY TO THROMBOSIS

Susceptibility to Thrombosis in Normal Young, Aging, Cortisone-treated, Heparinized and X-irradiated Hamsters as Tested by Topical Application of Thrombin

HERBERT J. BERMAN, GEORGE P. FULTON, BRENTON H. LUTZ and DAVID L. PIERCE