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Rabbit factor V: different effects of thrombin and venom, a source of error in assay[1]

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RAPAPORT, SAMUEL I., PETER F. HJORT, AND MARY JANE PATCH. *Rabbit factor V: different effects of thrombin and venom, a source of error in assay.* Am. J. Physiol. 211(6): 1477–1485. 1966.—Factor V was measured by one-stage techniques utilizing tissue thromboplastin and Russell viper venom on test samples of rabbit ear and heart blood prepared as diluted hemolyzed whole blood and as undiluted plasma. The assay with venom proved unreliable in the rabbit. It badly underestimates rabbit factor V activity relative to human factor V activity. Moreover, it grossly overestimates factor V activity in rabbit plasma treated with small amounts of thrombin or thought to contain traces of thrombin from its mode of collection, when such samples are compared with a standard rabbit plasma. Both of these discrepancies probably reflect the inability of rabbit factor V (in contrast to human factor V) to increase in reactivity on incubation with viper venom. Fresh (but not stored), diluted, hemolyzed ear blood samples assayed by the tissue thromboplastin technique proved satisfactory for serial factor V determinations in the rabbit with minimal blood loss.

assay of rabbit factor V; incubation of factor V with Russell viper venom; incubation of factor V with thrombin

THE RABBIT IS FREQUENTLY USED for studies involving diffuse intravascular clotting, and factor V is a sensitive indicator of this process. Hence, there is a need for a simple, reliable technique for serial measurement of rabbit factor V. Two one-stage factor V assays work well for human factor V—Owren's original method (11), which measures the ability of a test sample to shorten the tissue thromboplastin time of either hereditary factor V deficiency plasma or normal plasma depleted of factor V by storage; and the more recently described method of Borchgrevink, Pool, and Stormorken (2), which measures the ability of a test sample to shorten the clotting time of normal plasma depleted of factor V by treatment with a Russell viper venom-cephalin reagent.

The method with tissue thromboplastin measures the original activity state of factor V in the test sample. However, in the method with viper venom, native factor V (proaccelerin) increases in reactivity during the incubation interval in which the test sample is exposed to the substrate-venom mixture prior to recalcification. Hjort showed (6) that partially purified bovine factor V increases in reactivity on incubation with Russell viper venom in the absence of added calcium, and Borchgrevink and co-workers (2) attributed the increase in factor V activity during incubation in their assay to a similar direct activation of factor V by venom. Sometimes this phenomenon causes the venom and tissue thromboplastin methods to yield different values for factor V in human plasma. For example, if a human plasma test sample contains both native factor V and contaminating thrombin-altered factor V (the activity the Scandinavian workers meant by the name accelerin (6)), it will clot faster in the assay with tissue thromboplastin than if its factor V were all in the native state. Since the reference curve for converting clotting times to percent activity is made from a standard plasma containing only native factor V, the test sample will be read as possessing a higher percent factor V activity than it really does. In the venom assay, however, the native factor V in the human test sample will increase in reactivity during the incubation period to match the activity of the thrombin-altered factor V. Because the same increase in native factor V activity occurs in the standard plasma used for the reference curve, significant error due to contamination of the test sample with thrombin-altered factor V is said to be eliminated (2).

Therefore, we thought that the assay with venom would be the better test for measuring factor V during intravascular clotting in the rabbit, wherein the possibility of contamination of test samples with thrombin-altered factor V arises. However, serious discrepancies

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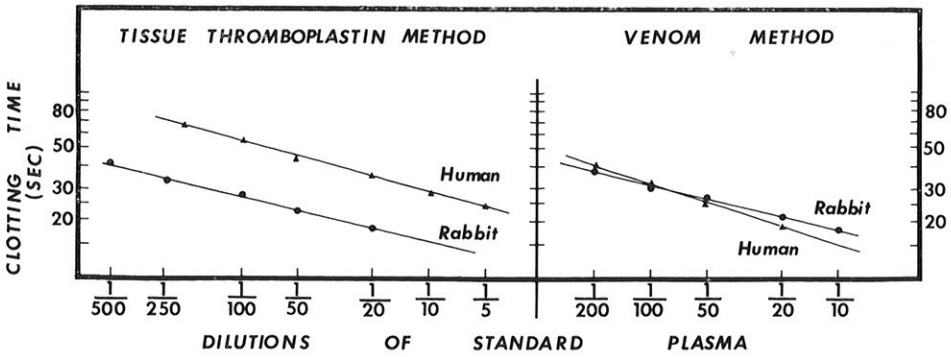

FIG. 1. Dilution curves for the factor V assay with tissue thromboplastin and the factor V assay with venom made with standard human and rabbit plasmas.

with the venom method were encountered in this species. Investigating their sources, we discovered that rabbit factor V does not increase in reactivity on incubation with Russell viper venom. At least partly as a consequence and in striking contrast to the experience with human plasma, the assay with viper venom is exquisitely sensitive to the effect of traces of thrombin in rabbit plasma test samples. These results are reported and discussed herein, along with practical suggestions for the assay of rabbit factor V.

MATERIALS AND METHODS

Materials

Citrate anticoagulant is a solution 0.06 M in sodium citrate and 0.04 M in citric acid.

Citrate diluting fluid contains one part of 0.1 M sodium citrate plus five parts of isotonic saline solution.

Barbital buffer (pH 7.35, ionic strength 0.15) is made as described elsewhere (14).

Tissue thromboplastin is a saline extract of human brain (12).

Substrate for the factor V assay with tissue thromboplastin. This is normal, oxalated human plasma depleted of factor V by incubation at pH 8.2 (16).

Substrate for the factor V assay with Russell viper venom. This is human plasma deficient in factor V and containing Russell viper venom and cephalin. It is made by mixing equal parts of platelet-poor citrated normal plasma and Russell viper venom 1:100,000 in cephalin suspension 1:100 as described by Borchgrevink et al. (2).

Bovine thrombin. Bovine thrombin (Topostasin, Hoffman-La Roche, Basle) is dissolved in oxalated saline, adsorbed with barium sulfate to remove impurities as described elsewhere (14), and stored frozen as a stock solution containing 500 NIH U/ml.

Human thrombin is a purified preparation (10) obtained through the generosity of Dr. Kent Miller of Albany, New York. When the contents of one ampule were dissolved in 3 ml distilled water, the solution contained, under our conditions of assay, between 60 and 70 NIH U/ml. It was stored frozen in a plastic vial at −20 C. Stock thrombin solutions were unfrozen and diluted in barbital buffer to the desired concentrations just prior to use.

METHODS

Preparation of Test Samples

Rabbit ear blood. The ear was shaved, rubbed gently with gauze moistened with xylene until the vessels were dilated, and then dried with gauze. A thin layer of Vaseline was applied to make a "nonwettable" surface. A 21-gauge needle was inserted rapidly into a vein to the end of the bevel and withdrawn. The first drop of blood was discarded.

Because one goal was to develop a technique for serial measurement of factor V with minimal blood loss, both "micro samples" of diluted whole blood and "macro samples" of plasma from ear blood were prepared. To prepare a micro sample, 0.05 ml of freely flowing blood was transferred with a capillary pipette to a 1-ml plastic vial containing 0.45 ml citrated diluting fluid. After mixing, the sample was frozen at −20 C and then either thawed within about 1 hr or stored frozen for stability studies. The freezing and thawing gave an evenly hemolyzed sample. The dilution of plasma in the test sample was calculated from the microhematrocrit, determined whenever a micro sample was drawn. Macro samples of plasma from ear blood were obtained by collecting rapidly flowing drops of blood in a plastic tube marked at 1 ml and containing 0.1 ml citrate anticoagulant. The plasma was removed after centrifugation for 10 min at 2,200 rpm and either used fresh or stored at −20 C in a 1-ml capped plastic vial.

Rabbit heart blood. Nine parts of blood were drawn into a plastic or silicone-treated syringe containing one part of citrate anticoagulant. The blood was transferred to a plastic tube, 0.05-ml subsamples were removed to prepare microsamples as described above, and the remainder was centrifuged to obtain plasma for macro samples. These were either used fresh or stored as described above.

Human venous blood. This was obtained from normal volunteers by collecting nine parts of blood through a "silicone-coated" needle into a plastic tube containing one part of citrate anticoagulant. The plasma was separated after high-speed centrifugation and either used fresh or stored at −20 C.

Assay Techniques

Factor V assay with tissue thromboplastin. To 0.1 ml of factor V-deficient substrate plasma were added 0.1 ml of tissue thromboplastin and 0.1 ml of a dilution of the test sample. After incubation for 3 min at 37 C, the mixture was recalcified with 0.1 ml of 40 mM calcium chloride solution.

Factor V assay with venom. To 0.2 ml of substrate plasma-venom mixture was added 0.1 ml of a dilution of the test sample. After incubation for exactly 3 min at 37 C, the mixture was recalcified with 0.1 ml of 35 mM calcium chloride solution.

Macro samples of rabbit plasma were usually diluted 1:80 just prior to assay. Micro samples, already diluted about 1:17 (the exact dilution varied with the hematocrit), were usually further diluted 1:5. All dilutions were made in citrated diluting fluid.

Reference curves to convert clotting times to percent activity were made with dilutions of citrated standard rabbit and human plasmas. The former was pooled plasma obtained from vena cava blood from three healthy rabbits and had been stored at −20 C for approximately 1 month. The latter was pooled plasma from five normal humans and had been stored at −20 C for approximately 3 weeks. (We have repeatedly stored citrated human plasma at −20 C for many weeks without measurable loss of factor V activity in either the tissue thromboplastin or venom assay.)

Factor X was assayed by a modification of Hougie's method (7) in which 0.1 ml of a mixture of equal parts of bentonite-adsorbed human plasma (prothrombin source) and barium sulfate-adsorbed ox plasma (factor V and fibrinogen source), 0.1 ml of the test reagent, and 0.1 ml of Russell viper venom 1:300,000 in cephalin suspension 1:50 were incubated together for exactly 3 min at 37 C and recalcified with 0.1 ml of 40 mM calcium chloride solution. The blank time with citrated diluting fluid was about 180 sec and the clotting time with a 1:10 dilution of citrated rabbit plasma was 23 sec.

Thrombin concentrations were determined by doing thrombin times on appropriate dilutions of the bovine and human thrombin preparations (0.2 ml plasma plus 0.1 ml diluted thrombin) and converting these times to NIH units from reference curves prepared with standard thrombin (Division of Biologic Standards, National Institutes of Health) as described in detail elsewhere (15).

Because the reference curves were logarithmic plots, log percent activity was used to calculate the means and standard deviation of factor V activity shown in the tables.

RESULTS

1. *The Different Relation Between Human and Rabbit Factor V Levels Obtained With Tissue Thromboplastin and Venom Methods*

Figure 1 is a plot of dilution curves made with our standard human and rabbit plasmas for the assay with tissue thromboplastin and the assay with venom. First, they clearly show that at any dilution rabbit test plasma clots much faster than human test plasma in the former assay but not in the latter. Second, they show that in each assay the slope of the dilution curve is less steep for rabbit plasma than for human plasma. This has been observed consistently and means that to the extent the slopes for the two species are not parallel neither assay can be used to define quantitatively the relation between human and rabbit factor V levels.

TABLE 1. *Factor V in rabbit plasma**

Reference Plasma Species	Factor V Activity in Percent†	
	Tissue Thromboplastin Assay	Venom Assay
Rabbit	102 (66–144)	152 (64–270)
Human	1,200 (880–1,500)	138 (78–215)

* Measured in the tissue thromboplastin and venom assays and converted to percent activity from reference curves made with standard rabbit plasma and standard human plasmas.
† Means and 1 SD, fresh heart plasma from nine animals.

Fresh rabbit plasma from heart blood was assayed for factor V by each technique in nine animals. The clotting times in each assay were converted to percent activity from the reference curves of Fig. 1. Percentages read from the rabbit plasma curves were slightly higher for the venom method but the two methods agreed reasonably well (first line of Table 1). In sharp contrast, when the same clotting times were converted to percentages from the human plasma curves, percent factor V obtained by the tissue thromboplastin method was about 10 times greater than percent factor V obtained by the venom method (second line of Table 1). Rabbit plasma contains much more factor V than human plasma (13), and our data from the assay with tissue thromboplastin indicate that the mean factor V level of rabbit plasma is about 12 times the mean factor V level of human plasma. Yet our data with the venom method show about equal factor V levels in human and rabbit plasma. This striking discrepancy suggested that rabbit factor V is much less responsive than human factor V to the conditions of the assay with venom. This was not remedied by adjusting the incubation period. Thus, prolonging the exposure of rabbit test plasma to substrate-venom mixture shortened its clotting time in the venom assay only slightly (e.g., with incubation times of 3, 6, 9 and 12 minutes, a 1:80 dilution of rabbit plasma gave clotting times of 39, 37, 36, and 37 seconds, respectively).

2. *Insensitivity of Rabbit Factor V to Russell Viper Venom*

If rabbit V were insensitive to venom, then, in converting clotting times of rabbit test plasma in the venom method to percentages from dilution curves made from human plasma, one could be comparing native rabbit factor V with human factor V which had increased in

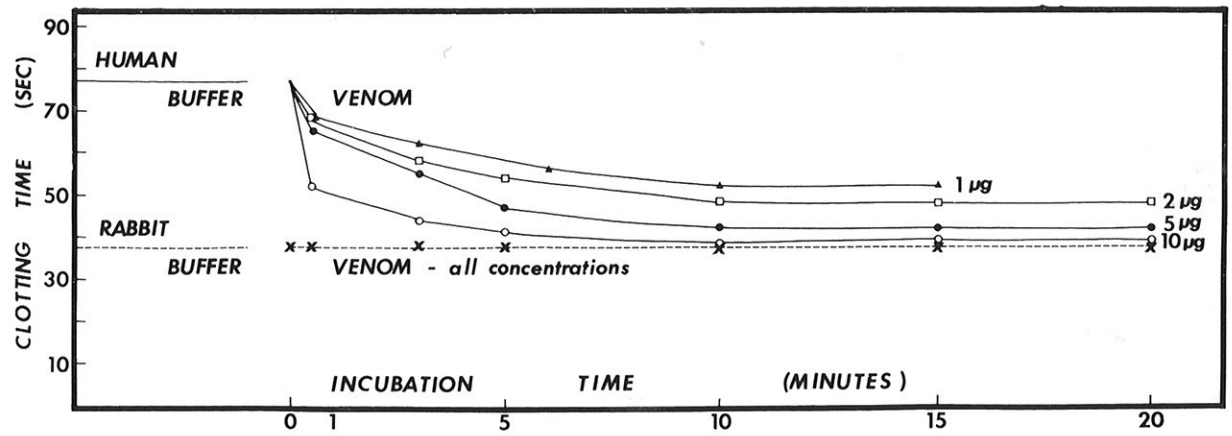

FIG. 2. Ability of venom to increase the reactivity of factor V in adsorbed human plasma but not in adsorbed rabbit plasma. At room temperature one part of buffer (control) or venom was added to nine parts of oxalated, barium sulfate-adsorbed human or rabbit plasma to give the final venom concentrations shown in the figure (μg/ml). At the incubation times shown, subsamples were diluted in citrated diluting fluid (1:50 for human and 1:100 for rabbit plasma) and assayed for factor V activity by the tissue thromboplastin technique, which was modified by adding the test sample just before the calcium. The clotting time with buffer has been taken as the clotting time at zero incubation time for a subsample from the incubation mixtures containing venom. The first subsample from such a mixture, taken as quickly as possible after adding the venom to the plasma, has been plotted at an incubation time of ½ min.

reactivity during the process of assay. This could mask a large difference in the native factor V content of rabbit and human plasma.

To compare the direct effect of venom on human and rabbit factor V, we first adsorbed fresh oxalated human and rabbit plasmas with barium sulfate powder (50 mg/ml for 10 min at 4 C, adsorbed a second time for rabbit plasma) to remove factor X (blank times of the factor X assay, 160–195 sec; clotting times of a 1:5 dilution of adsorbed human plasma, 155–185 sec; clotting times of a 1:5 dilution of adsorbed rabbit plasma, 140–160 sec). It is now well established that Russell viper venom will activate factor X in the presence of calcium (5, 8) and there is recent evidence that such activated factor X will in turn activate factor V (3). Therefore, this preliminary adsorption was essential to assure ourselves that any increase in factor V reactivity found on incubation of such oxalated, factor X-free plasmas would truly represent a direct effect of venom on factor V.

Adsorbed human and rabbit plasmas were incubated at room temperature with either barbital buffer (control) or various concentrations of venom in barbital buffer. Serial subsamples were diluted and assayed for factor V activity in the assay with tissue thromboplastin, which was modified by adding the test plasma just before the calcium to reduce the possibility of the diluted test sample interacting with the substrate-tissue thromboplastin mixture prior to recalcification.

Figure 2 is a plot of data obtained when the final concentration of venom in the incubation mixture was varied between 1 and 10 μg/ml. The behavior of human and rabbit plasma differed strikingly; venom clearly increased the reactivity of human factor V but not of rabbit factor V. All venom-rabbit plasma mixtures gave exactly the same clotting times as the buffer-rabbit plasma control mixture. Similar data (not shown to simplify Fig. 2) were obtained when the venom concentration was increased to 20 μg/ml, which is six times the concentration of venom in the incubation mixture of the venom assay (3.3 μg/ml).

Figure 2 also shows that the venom concentration determined the degree of shortening obtained. The clotting times of human plasma incubated with different concentrations of venom leveled off and did not approach each other, which suggested that the venom was being used up in the reaction. When the shortest clotting time for each venom concentration was converted to percent activity from a reference curve made with adsorbed plasma incubated with control buffer, the percentages listed in Table 2 were obtained. These data indicate that the venom does not act enzymatically to increase human factor V reactivity but that the increased reactivity stems from the formation of a complex between venom and human factor V.

3. Evidence That Increased Reactivity of Factor V After Incubation in Venom Assay Stems From More Than a Direct Action of Venom on Factor V

Venom cannot increase the reactivity of rabbit factor V in oxalated, adsorbed plasma. Therefore, rabbit factor V should not increase in reactivity during incubation in the venom assay if this increased reactivity stems solely from a direct action of venom on factor V. Yet, as Table 3 shows, rabbit test plasma, like human test plasma, clots more rapidly in the venom assay when the test plasma and substrate-venom mixture are incubated together than when they are incubated separately and combined just before recalcification. A mechanism in addition to direct activation by factor V must be postulated.

TABLE 2. *Percent factor V activity**

Final Venom concn in Incubation Mixture, μg/ml	Clotting Time of 1/50 Dilution, sec	Factor V Percent Activity†
20	35	2,700
10	38	2,100
5	42	1,400
2	48	800
1	52	575

* Measured by the tissue thromboplastin method in human adsorbed plasma incubated with various concentrations of venom. †Calculated from curve made with a control adsorbed plasma-buffer incubation mixture. Clotting time of a 1/5 dilution, 41 sec.

Further evidence for this second mechanism was obtained in experiments in which subsamples from an incubation mixture containing either adsorbed rabbit plasma or adsorbed human plasma and venom were clotted in the venom assay. These subsamples were also clotted in two ways: by incubating the test plasma and substrate-venom mixture together and by adding the test plasma just prior to recalcification. Thus, each test sample was exposed to venom in a preliminary incubation mixture and one-half of the samples underwent an additional incubation with the substrate-venom mixture. The data from one experiment are summarized in Table 4 and show the following:

1) Adsorbed rabbit plasma incubated with venom (final concn 10 μg/ml) for 10 min gave the same clotting times as adsorbed rabbit plasma mixed with control buffer, if the test sample was added to the clotting system just prior to recalcification. If, however, the test sample was allowed to incubate further with the substrate-venom mixture (final venom concn 3.3 μg/ml), its clotting time shortened significantly. Thus, incubation with substrate-venom increased factor V activity in an adsorbed rabbit plasma sample proven to resist the action of venom alone.

2) Adsorbed human plasma clotted faster after incubation with venom than after incubation with control buffer, when the test plasma was added to the substrate-venom just before recalcification. The venom appeared to act very quickly. As repeatedly observed, the subsample taken immediately after adding the venom clotted just as rapidly as the subsample taken after 10 min of incubation. When the test samples were further incubated with the substrate-venom mixture, a second increase in factor V activity was demonstrated.

These data indicate that the substrate-venom mixture contains an activity different from venom, active in the absence of added calcium, and capable of acting on both native rabbit factor V and human factor V which has been altered by exposure to venom alone. Breckenridge and Ratnoff (3) found that activated factor X will activate factor V in the presence of citrate ions. Since citrate is the only decalcifying agent present in significant amounts in the venom assay, traces of activated factor X could account for this incubation effect of the substrate-venom mixture.

4. A Possible Explanation for the Different Reactivity of Rabbit and Human Factor V in the Venom Assay

The data obtained by the tissue thromboplastin method indicate that rabbit plasma contains about 12 times the factor V activity of human plasma (Table 1). Yet, as Table 3 shows, a 1:80 dilution of rabbit plasma—not a 1:1200—gave the same clotting time (40 sec) as a 1:100 dilution of human plasma in the venom assay when the test plasma was added just before the calcium. Thus, the relative insensitivity of the assay to rabbit factor V persists when effects secondary to the 3-min incubation of test plasma with substrate-venom are eliminated. This fits with the observation (Tables 3 and 4) that rabbit and human factor V do not differ in their ability to increase in reactivity on incubation with an unknown activity (possibly activated factor X) in the substrate-venom mixture.

However, rabbit plasma does differ strikingly from human plasma in failing to increase in reactivity on exposure to venom alone. Therefore, the question arises—does venom act on human factor V fast enough to account for the different sensitivity of the assay to rabbit and human plasma still evident when the test plasma is added just before recalcification? As Table 4 shows, the full effect of venom (final concn 10 μg/ml) on human plasma was manifest in a subsample taken immediately after adding the venom to the plasma. However, in that experiment the subsample stood for 3 min after its dilution before being added to the clotting mixture. When this additional interval was eliminated, i.e., when one part of venom was added to nine parts of adsorbed human plasma (final concn 10 μg/ml) and a subsample was taken immediately, diluted, added as quickly as possible to the warmed substrate-venom mixture, and the mixture recalcified—an effect of venom was still demonstrable. For example, in one experiment the clotting time of an adsorbed plasma-control buffer mixture was 63 sec and of an adsorbed plasma-venom mixture, 56 sec. However, when the venom concentration in the adsorbed plasma-venom mixture was reduced to 3.3 μg/ml, i.e., to the same final concentration of venom as in the incu-

TABLE 3. *Shortening of clotting time in venom assay when either rabbit or human test plasma is incubated with the substrate-venom reagent*

Test Sample	Dilution of Test Sample	Clotting Times, sec, in Venom Assay When Test Plasma Was Incubated	
		Separately*	With Substrate-Venom†
Human plasma	1:100	40	30
Rabbit plasma	1:80	40	31
Diluting fluid			73

* Test sample and substrate-venom reagent incubated separately for 3 min at 37 C and combined just prior to recalcification. † Test carried out as described under METHODS.

TABLE 4. *Effect of a preliminary incubation of adsorbed rabbit and human plasma with venom on clotting times in venom assay**

Test Plasma†	Mixed With	Subsampled After, min	Clotting Times, sec, in Venom Assay When Test Plasma Was Incubated	
			Separately	With Substrate-Venom
Ads. rabbit plasma	Buffer		71	51
	Venom	<½	70	52
		5	68	51
		10	70	51
Ads. human plasma	Buffer		65	40
	Venom	<½	50	
		5	51	43
		10	50	44
Buffer			180	180

* When test plasma is and is not incubated with substrate-venom mixture prior to recalcification. † Adsorbed human or rabbit plasma (0.9 ml) was mixed with 0.1 ml buffer or venom in buffer (final concn 10 μg/ml) at room temperature. At the times indicated (<½ min means as quickly as possible after reagents mixed), subsamples were diluted 1/50 (human) or 1/100 (rabbit) in citrated diluting fluid and assayed as described in footnote to Table 3.

bation mixture of the venom assay, a clear-cut immediate effect of venom could no longer be demonstrated. This can be interpreted in two ways: either venom at this concentration has no immediate effect on human factor V, or the immediate effect of venom at this concentration is masked by the effect of the same concentration of venom in the assay mixture acting on both the control and the experimental test samples. Thus, although the experiments are not conclusive, the possibility remains that the relative insensitivity of the venom assay to rabbit factor V reflects the inability of venom to act directly on rabbit factor V in contrast to its rapid direct effect on human factor V.

5. *Differences in Apparent Factor V Levels in Ear and Heart Samples Assayed by Tissue Thromboplastin and Venom Methods*

Ear and heart blood samples from the same animals were assayed for factor V by the tissue thromboplastin and venom methods, and clotting times were converted to percent activity from the dilution curves with standard rabbit plasma shown in Fig. 1. The values obtained with the tissue thromboplastin method are summarized in the upper part of Table 5. Since heart plasma (heart macro samples) should provide the most reliable sample for determining the true factor V level of an animal, it was encouraging to find that the mean for the ear micro samples differed very little from the mean for the heart macro samples. However, the mean for the ear macro samples was significantly higher, a finding compatible with nascent clotting during the collection of the ear macro samples.

TABLE 5. *Factor V in ear and heart blood samples as determined by tissue thromboplastin and venom assays*

Assay	Factor V Activity, Percent			
	Ear blood		Heart blood	
	Micro sample	Macro sample	Micro sample	Macro sample
Tissue thromboplastin	135* (103–177)	183 (145–232)	106† (79–142)	115* (85–155)
Venom	482 (288–641)	338 (192–595)	107† (67–171)	119 (66–215)

Values are means and 1 SD. Reference curves from standard rabbit plasma. * Means of nine animals, all other means are of six animals. † For micro samples from heart blood, 10% of the percentage actually measured has been added to give the final percentage shown. This corrects for the citrate anticoagulant in the heart blood (see METHODS).

These results contrast strikingly with the data shown in the lower part of Table 5, which are the means obtained by the venom method. With this technique ear blood, prepared either as micro or macro samples, appeared to contain up to four times more factor V than heart blood.

If these very high values for factor V in ear blood by the venom technique stemmed from nascent clotting of ear blood, then heavy anticoagulant therapy should reduce these values. Three rabbits were given daily intravenous injections of 30 mg sodium warfarin (Marevan, Nyco, Oslo) for 4 days and on the 5th day (when prothrombin times by the P-P method were about 1 %) ear and heart blood samples were assayed for factor V. The data are shown in Table 6. Clearly, anticoagulant therapy all but eliminated the difference between the values for factor V in ear and heart blood by the venom method. Moreover, the means in these anticoagulated animals, particularly mean factor V in heart blood by the venom method, were lower than our pooled rabbit plasma standard. This leads us to suspect that our standard contained a trace amount of factor V of increased reactivity, for, as will be shown later, the elevated levels of factor V in plasma from ear blood persist in samples stored at −20 C.

TABLE 6. *Factor V in ear and heart blood samples from three rabbits given sodium warfarin*

Assay	Mean Factor V Activity, Percent			
	Ear blood		Heart blood	
	Micro sample	Macro sample	Micro sample	Macro sample
Tissue thromboplastin	95	89	76	73
Venom	94	68	57	59

Reference curves from standard rabbit plasma.

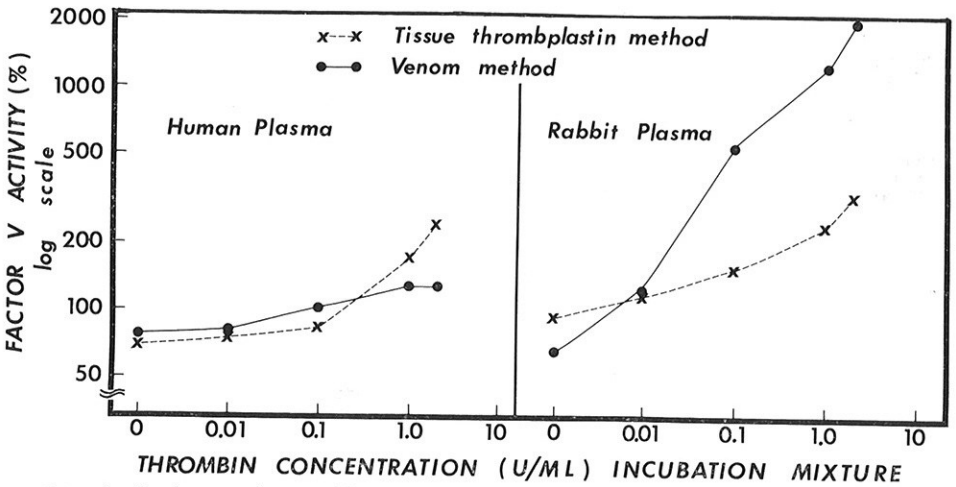

FIG. 3. The striking sensitivity of the venom assay to thrombin-altered rabbit factor V. To 0.9-ml aliquots of human or rabbit plasma was added 0.1 ml barbital buffer (control) or adsorbed bovine thrombin in barbital buffer to give the final thrombin concentrations shown. Two, five, and eight minutes later, subsamples were diluted in citrated diluting fluid, and their factor V activity was measured in the tissue thromboplastin and venom assays. Values shown are means of the three determinations at each thrombin concentration. (The highest thrombin concentration added to the clotting system was one-half of that capable of influencing the blank time of the tissue thromboplastin assay.) Clotting times with human plasma were converted to percent from reference curves made with standard human plasma; clotting times with rabbit plasma were converted to percent from reference curves made with standard rabbit plasma.

6. Evidence of Sensitivity of the Assay With Venom to the Effect of Incubating Rabbit Plasma With Small Amounts of Thrombin

Why did the assay with venom yield so much higher values for factor V in ear blood than the assay with tissue thromboplastin? Since an activity arising during nascent clotting appeared to be responsible, it seemed important to determine if this activity was thrombin itself. Borchgrevink and co-workers had reported (2) that the venom technique minimized error due to exposure of human factor V to thrombin. But because venom fails to act directly on rabbit factor V, the possibility arose that the opposite might be found in this species and that the assay with venom might be peculiarly sensitive to thrombin-altered rabbit factor V.

To compare the sensitivity of the assay with venom and the assay with tissue thromboplastin to thrombin-altered human and rabbit factor V, we incubated human plasma and rabbit plasma (from vena cava blood) with increasing amounts of adsorbed bovine thrombin and assayed the resultant activities by the two techniques. The data from one such experiment are plotted in Fig. 3. Note first that adding adsorbed bovine thrombin to human plasma (in a final concn of up to 3 NIH U/ml) increased its factor V activity more than threefold as measured in the assay with tissue thromboplastin but only slightly as measured in the assay with venom. Thus, thrombin carried over with the diluted test plasma into the clotting mixture could have had little or no effect on the venom assay itself. The data confirm the observation of Borchgrevink and co-workers (2) that the venom assay is less sensitive than the tissue thromboplastin assay to thrombin-altered human factor V.

Just the opposite was found with rabbit plasma. As Fig. 3 shows, as little as 0.1 NIH units of thrombin per milliliter (final concn) caused a striking rise in factor V activity as measured in the assay with venom (to 480%) but only a slight rise in factor V activity as measured in the assay with tissue thromboplastin (to 153%).

Although the bovine thrombin used in these experiments had been adsorbed with barium sulfate powder to remove contaminants, it seemed important to confirm that the effect observed was due to thrombin and not to an active intermediate in the thrombin preparation, e.g., activated factor X. Therefore, in a further experiment, adsorbed human and rabbit plasmas were incubated with a preparation of highly purified human thrombin (10) in a final concentration of 0.1 U/ml. (At this dilution the thrombin had no measurable effect on our factor X assay: blank time, 175 sec; clotting time of a test sample of thrombin, 0.1 U/ml, 178 sec.) The clotting times found when subsamples were assayed in the unmodified venom assay are listed in Table 7. These data confirm the striking sensitivity of the assay with venom to the increased reactivity which follows the exposure of native rabbit factor V to traces of thrombin. They strongly support the conclusion that the very high values for factor V in ear blood by the venom method stemmed from slight contamination of ear blood samples with thrombin-altered factor V.

7. Factor V Activity in Stored Micro and Macro Samples

Because fresh ear micro and heart macro samples agreed reasonably well in the assay with tissue thromboplastin (see Table 5), it was of practical importance to determine if factor V activity would hold up in ear micro samples stored at −20 C. Unfortunately, as little as 2½ hr of storage led to a small but definite loss of activity. Thus, mean and one standard deviation for factor V activity in micro samples from six animals in the assay with tissue thromboplastin were as follows: samples not frozen and assayed within ½ hr, 138 (111–171) %; samples stored frozen for 1 hr, 121 (99–149) %; samples stored frozen for 2½ hr, 108 (90–129) %; samples frozen for 4 hr, 108 (94–121) %.

Table 8 summarizes data illustrating the effect of longer periods of storage in both assays. The considerable loss of factor V activity in ear micro samples in the assay

TABLE 7. *Increased factor V activity measured in venom assay when adsorbed human and rabbit plasma were incubated with purified human thrombin*

Test Material	Incubated With	Sub-sampled After, min	Factor V Activity in Venom Assay	
			Clotting times, sec	Activity,* %
Ads. human plasma	Buffer	2	58	100
		5	58	100
		8	57	110
	Thrombin	2	50	170
		5	48	190
		8	48	190
Ads. rabbit plasma	Buffer	2	47	130
		5	47	130
		8	49	110
	Thrombin	2	28	1,050
		5	24	2,000
		8	25	1,650
Diluting fluid	Buffer	2	180	
		8	180	
	Thrombin	2	180	
		8	180	

At room temperature 0.1 ml buffer (control) or purified human thrombin (1 NIH U/ml) was added to 0.9 ml adsorbed oxalated human or rabbit plasma. At the times indicated subsamples were diluted 1:100 in citrated diluting fluid and assayed for factor V activity in the unmodified venom assay. * Human test plasma samples read from a dilution curve made from a standard containing 9 parts of adsorbed human plasma plus 1 part of buffer. Rabbit plasma test samples read from an adsorbed rabbit plasma curve prepared in the same manner.

with tissue thromboplastin after 1 day of storage and the striking loss of activity in the assay with venom after 3 days of storage are readily apparent. Clearly, factor V activity in diluted hemolyzed ear blood does not withstand storage at −20 C. In contrast, macro samples of either ear or heart blood lost little and sometimes no activity on storage for 6–14 days at −20 C. Note that the striking difference in the assay with venom between factor V levels in fresh macro samples of ear and heart blood persisted when the samples were assayed again after 7 days of storage. If, as we believe, this difference stems from small amounts of contaminating thrombin-altered factor V in the ear samples, then thrombin-altered factor V must withstand storage at −20 C in undiluted rabbit plasma.

DISCUSSION

We have found two discrepancies when the venom technique is used to measure rabbit factor V. First, the venom technique badly underestimates the level of factor V in rabbit plasma when it is compared to the level of factor V in human plasma. Second, the venom technique grossly overestimates the factor V activity in rabbit plasma treated with small amounts of thrombin or thought to contain traces of thrombin from its mode of collection, when such test samples are compared to a standard rabbit plasma. This makes the venom assay unreliable in the rabbit.

Incubation with venom increases the reactivity of factor V in oxalated, barium sulfate-adsorbed human plasma but fails to increase the reactivity of factor V in similarly prepared rabbit plasma (Fig. 2; Tables 2, 4). This direct effect of venom on human factor V is not enzymatic but appears to result from the formation of a complex between venom and human factor V. Borchgrevink and co-workers (2) attributed the increase in reactivity of human factor V during the incubation interval of the assay with venom to a direct effect of venom on factor V. However, both human and rabbit plasma test samples increase in reactivity on incubation in the venom assay (Table 3). Since rabbit factor V is insensitive to venom alone and since human factor V incubated with venom alone undergoes a further increase in reactivity on incubation in the assay with venom (Table 4), direct activation by venom cannot be the sole mechanism for the effect of incubation in the venom assay.

Our data are compatible with but do not prove (see *section 4* of RESULTS) the following hypothesis for the difference in sensitivity of the venom assay to human and rabbit factor V:

1) In human test plasma two increases in factor V reactivity occur in the venom assay prior to recalcification: a rapid direct effect of venom on factor V and a slower effect due to another activity, possibly activated factor X, present in the substrate-venom reagent.

2) In rabbit test plasma the venom cannot act directly on factor V. The increase in factor V reactivity prior to recalcification stems entirely from the second effect of the substrate-venom reagent.

Some investigators have maintained that thrombin can increase the reactivity of factor V (1, 6, 14, 18) but other investigators have been unable to confirm this (3, 17) and the point has remained in doubt. In the present study, rabbit plasma treated with small amounts of thrombin exhibited a remarkable increase in factor V reactivity in the venom assay. This resulted from an action of thrombin itself, and not of a contaminant such as activated factor X in the thrombin preparations, for it was demonstrable not only with barium sulfate-adsorbed commercial bovine thrombin but with a preparation of highly purified human thrombin without measurable factor X activity. Moreover, the effect was observed

TABLE 8. *Different storage stability of factor V in micro and macro samples*

Assay	Sample	No. of Animals	Days Stored	Factor V,* %
Tissue thrombo-plastin	Micro-ear	6	0	142
			1	83
	Macro-ear	6	0	152
			6–13	127
	Macro-heart	9	0	100
			6–14	100
Venom	Micro-ear	3	0	550
			3	58
	Macro-ear	6	0	304
			7	302
	Macro-heart	6	0	124
			7	92

* Means. Reference curve is standard rabbit plasma.

when this purified thrombin was added to oxalated, barium sulfate-adsorbed rabbit plasma, i.e., to factor V in the absence of a source of factor X or of ionized calcium. Since thrombin carried over into the clotting mixture had little if any influence on the venom assay itself (see *section 6* of RESULTS and Table 7), we conclude that the increase in factor V reactivity measured in the venom assay represents a direct effect of thrombin on rabbit factor V. Apparently, the venom assay is exquisitely sensitive to the effect of thrombin on rabbit factor V because rabbit factor V is insensitive to the direct effect of venom. The direct effect of venom on human factor V is thought to mask in large part the effect of incubating human factor V with thrombin (see Fig. 3 and Table 7).

Because of the possibility of contamination of samples with traces of thrombin, one should only use the tissue thromboplastin method to measure rabbit factor V. If just one or two determinations are needed, plasma from heart blood provides the best test sample, for it is least likely to contain thrombin-altered factor V and its factor V activity withstands storage at −20 C. Factor V in plasma from ear blood also withstands storage, but, at least by our technique of collection, many samples will contain enough thrombin-altered factor V to elevate the factor V level significantly even by the tissue thromboplastin technique.

Micro samples of diluted, hemolyzed ear blood possess three advantages for repeated determinations of factor V in the rabbit: ease of sampling, negligible blood loss, and elimination of the risk of fatal cardiac tamponade (a real risk when intravascular clotting induces marked consumption of clotting factors and platelets). Although such samples do contain thrombin-altered factor V as demonstrated by the assay with venom, the error in the assay with tissue thromboplastin is usually small (see Table 5). Moreover, since multiple samples are assayed, an individual sample grossly in error can be recognized. The major drawback of the micro sample is that it cannot be stored. It must be frozen and thawed (to give an even, hemolyzed sample) and assayed within about 1 hr. Handled in this way, micro samples of ear blood assayed by the tissue thromboplastin technique will furnish serial factor V measurements in the rabbit satisfactory for most purposes.

Finally, one must not confuse the increased reactivity of factor V induced by thrombin and induced by venom with the activation of factor V by activated factor X and lipid in the presence of calcium ions during the final step of the generation of the intrinsic prothrombin activator (4, 9). The former increases in reactivity do not bypass or substitute for the latter; rather, they appear to facilitate and, under some circumstances, may be a prerequisite (1) for the latter. Russell viper venom in the presence of calcium has been used to activate factor X in recent important studies of the intrinsic clotting reactions (3, 5, 8). In one such study (3), a mixture of activated factor X and venom was incubated with human factor V and it was implied that the activated factor X activated native human factor V. In interpreting such data we believe it is important to be aware that the venom in the mixture also reacts with factor V and that the result observed stems from a combined effect of both venom and activated factor X on human factor V.

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