Influence of Organ Extracts on Blood Clotting Factors

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Organs damaged by trauma or pathological changes have a profound influence on blood and damaged tissues often induce rapid clotting in vitro, which is followed by fibrinolysis (1).

Experimental introduction of tissue with thromboplastic activity into the circulation produces intravascular clotting which may be followed by bleeding due to consumption of clotting factors in the clotting process. A similar mechanism probably operates in comparable pathological states (2—6). It is also known that the tissue thromboplastins are inactivated by serum (7, 8). The reaction of thromboplastic material with the plasma clotting components leads to the production of serum factors, some of which are very active in promoting clotting (9).

We wished to study the clotting activity of serum in thrombotic conditions, in which blood is presumably in contact with damaged tissues. As part of such an investigation, experiments were carried out in order to study the influence of tissue extracts on clotting factors in vitro and the results are reported here.

Materials and Methods

Venous blood from healthy donors was used. Serum was obtained from blood collected in a tube containing glass wool and allowed to clot at 37° C for 1½ hours. Human brain was dehydrated with acetone (10).

For most of the experiments the starting material was a 6% suspension of dehydrated human brain in 0.145 M NaCl. The suspension was kept at 37° C for 1½ hours, the brain particles being ground in the tube with a pestle until a very fine suspension was obtained.

Rabbit's brain and lung were removed under nembutal anaesthesia and ground in a blendor with an equal volume of 0.145 M NaCl.

Euglobulins were prepared from serum by dilution with 10 volumes of ice-cold distilled water; 1 drop of sec. octyl alcohol was then added to prevent foaming and carbon dioxide was bubbled through the mixture at 4° C for 5 minutes. The sediment after centrifugation wa:

*) This investigation was supported by a grant from the National Health and Medical Research Council of Australia.

dissolved in half of the original serum volume of 0.145 M NaCl with $NaHCO_3$ added to pH 7.4.

Phospholipid (P-lipid) was prepared by ether extraction of dehydrated brain powder (11); suspensions were prepared in veronal buffer, pH 7.4.

The thromboplastin generation test (12) was carried out at 28° C with barium sulphate-treated plasma (Ba-plasma). Prothrombin was assayed (13). Factor VII activity was determined (14). The required brain extract was prepared by centrifugation of the 6% human brain suspension at 2000 g (calculated for the bottom of the tube), for 30 minutes at 4° C. The supernatant was used; this was a very active and stable preparation. To 0.1 ml of a 10-fold dilution of plasma in Ba-plasma was added 0.01 ml of the serum to be tested and 0.2 ml of a mixture of equal volumes of brain extract and 0.01 M calcium chloride at 37° C. This test gave satisfactory results with sera which contained 0—5% prothrombin.

Sera from with prothrombin was absent could be tested more accurately by incubating 0.2 ml of brain-calcium chloride mixture and 0.01 ml serum at 37° C for 3 minutes and observing clotting after addition of 0.1 ml 10% plasma in Ba-plasma.

Factor IX activity of sera was determined with the following technique:

Serum was obtained from blood of a patient with a congenital complete factor IX deficiency.

To 0.06 ml of this serum was added 0.01 ml of the test serum and a thromboplastin generation test was carried out. Standard curves were obtained from mixtures of diluted normal human sera and the patient's serum.

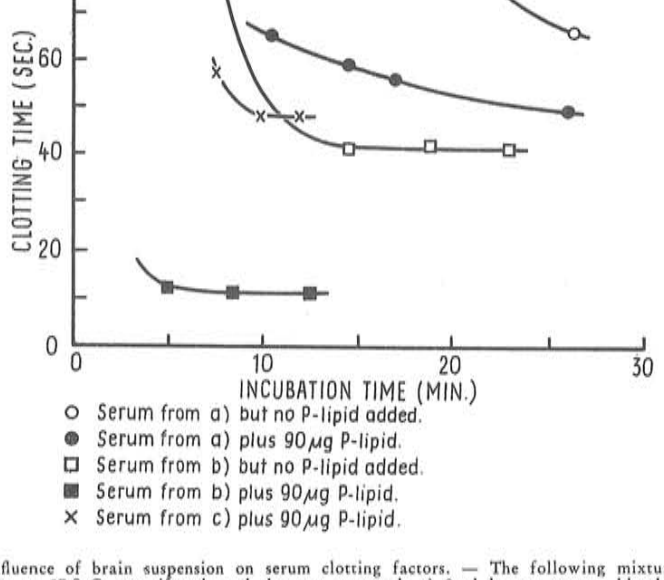

Fig. 1: Influence of brain suspension on serum clotting factors. — The following mixtures were incubated 1 hour at 37° C, centrifuged, and the sera removed. a) 8 ml human venous blood mixed with 2 ml homologous brain suspension; b) 8 ml human venous blood mixed with 2 ml 0.145 M NaCl; c) 2 ml human serum mixed with 0.5 ml homologous brain suspension. Thromboplastin generation test was performed with 0.12 ml normal human Ba-plasma, 0.1 ml of serum, 0.6 ml 0.025 M $CaCl_2$, and other additions as indicated; 0.145 M NaCl was added to final volume of 2.4 ml. The P-lipids added were isolated from human brain as under Materials and Methods.

Lipid phosphorus was determined in the trichloroacetic acid insoluble fraction (15). P × 25 was taken as the weight of P-lipid.

The pH of the reaction mixtures was 7.6—7.7.

Results

Venous blood or serum was incubated with homologous brain suspension. The sera obtained were tested for their ability to take part in thromboplastin formation. Results are shown in figure 1.

The results indicate that the experimental sera showed a much diminished activity in the production of blood thromboplastin.

Further experiments were carried out to find the cause of the inactivity of the serum clotting factors. First it was determined whether the brain extract interfered with thromboplastin formation or whether an inhibitor of thromboplastin was produced in the reaction of brain extract with blood or serum. Results of relevant experiments are given in table 1 from which it is seen that in the formation of blood thromboplastin the brain suspension was quite an effective substitute for free P-lipids in amounts which produced inactive sera in figure.

It is unlikely that the brain preparations contained all the trichloroacetic acid-insoluble phosphorus as free P-lipids. Experiment J indicates further that the amount of brain thromboplastin transferred into substrate plasma cannot

Table 1: Brain Extract as Substitute for P-lipids in Formation of Blood Thromboplastin

Composition of Reaction Mixture*)	Clotting Time (sec.)	Incubation Time (min.)
A. Normal human Ba-plasma and serum. No P-lipids added	36	12
B. The same as A plus P-lipids containing 10 µg lipid P	10	5—8
C. The same as A plus P-lipids containing 5 µg lipid P	11.5	6
D. The same as A plus 0.1 ml 6% human brain suspension	12	< 4
E. The same as A plus 0.05 ml 6% human brain suspension	12	< 4
F. The same as A plus 0.02 ml 6% human brain suspension	12	4
G. The same as A plus 0.01 ml 6% human brain suspension	13.5	5
H. The same as A plus 0.005 ml 6% human brain suspension	15.5	4
J. 0.06 ml normal human serum plus 0.03 ml 6% human brain suspension	27	< 3

*) The thromboplastin generation test was carried out similarly to that given in Fig. 1. The figures recorded are the shortest clotting times, and the shortest incubation times required for development of maximum activity. The brain suspension contained 170 µg of trichloroacetic acid-insoluble P per ml.

cause the short clotting times observed in the experiments D to H. It is therefore apparent that the original brain extract did not contain inhibitors of blood thromboplastin.

Further, it was determined whether sera obtained after incubation of blood with brain extracts were inhibitory when added to untreated sera. Results are given in table 2.

Table 2: Influence of Brain Extract on Serum Clotting Factors*)

	Clotting Time (sec.)	Incubation Time (min.)
A. 0.06 ml serum from 8 ml venous blood incubated with 2 ml 6% human brain suspension 90 min., 37° C	24	8
B. 0.06 ml serum from 8 ml venous blood incubated with 2 ml 0.145 M NaCl 90 min., 37° C	10.5	< 3
C. 0.06 ml euglobulin prepared from serum A	27	8
D. 0.06 ml euglobulin prepared from serum B	10.5	5
E. 0.06 ml serum A + 0.006 ml serum B	14	9
F. 0.06 ml euglobulin C + 0.006 ml serum B	15	5
G. 0.01 ml serum B	12.5	7
H. 0.006 ml serum B	17	10

*) The thromboplastin generation test was carried out similarly to that in Fig. 1. Sera and euglobulin fractions were free of prothrombin. The figures given are shortest clotting times, and shortest incubation times required for development of maximum activity.

The results given in table 2 indicate that sera obtained after incubation of brain suspension with blood or serum have activities less than 10% of that of untreated serum. The diminished activity is not due to an inhibition of thromboplastin formation, because addition of untreated serum to the deficient serum resulted in thromboplastin formation as expected from an uninhibited mixture. Additional support for the absence of inhibitors is given by the euglobulin experiments (table 2), since inhibitors of thromboplastin formation are usually absent from the euglobulin fraction of serum (16).

Therefore it is most probable that the brain extracts inactivated one or more components required for thromboplastin formation.

In the following experiments the sera from brain-blood incubation mixtures were assayed for factor IX activity.

It is seen from the results given in table 3 that the factor IX activity of the experimental sera is very low. The clotting defect could not be corrected by

Table 3: Factor IX Activity of Serum Obtained after Incubation of Blood or Serum with Brain Extract*)

	Clotting Time (sec.)	Incubation Time (min.)	Factor IX Activity in % of normal
A. 0.06 ml serum from patient with complete factor IX deficiency	40	9	0
B. 0.06 ml serum after incubation of 1 ml 6% human brain suspension with 4 ml normal human venous blood, 75 min., 37° C	> 60	18	< 10**)
C. 0.06 ml serum after incubation of 1 ml 0.145 M NaCl + 4 ml normal human venous blood	12	7	100
D. 0.06 ml serum after incubation of 1 ml 6% brain suspension + 4 ml normal human serum	48	10	15**)
E. 0.06 ml serum after incubation of 1 ml 0.145 M NaCl + 4 ml normal human serum	10	< 5	100
F. 0.06 ml serum A + 0.006 ml serum C	11.5	< 5	10
G. 0.06 ml serum A + 0.002 ml serum C	14.5	< 3	3.3
H. 0.06 ml serum A + 0.01 ml serum B	26	7	—
J. 0.06 ml serum A + 0.01 ml serum D	17	7	—
K. 0.06 ml Marcoumar serum	17.5	14	—
L. 0.01 ml Marcoumar serum + 0.05 ml serum B	27	16	—
M. 0.01 ml serum C + 0.05 ml serum B	19	15	—

*) The thromboplastin generation test was carried out in similar manner to that described in Fig. 1. Figures recorded are the shortest clotting times, and the shortest incubation times required for development of maximum activity.

**) These figures were calculated by comparing results of experiments H and J with those of experiments A, G, and F in which various mixtures of normal and factor IX-deficient serum were tested.

Table 4: Factor VII Activity of Serum Obtained after Incubation of Blood or Serum with Brain Extract*)

	Clotting Time (sec.)	Factor VII Activity in % of normal
A. 10% normal human plasma in Ba-plasma	36—48	0
B. The same as A plus 0.01 ml normal human serum	21—24	100
C. The same as A plus 0.01 ml serum I*)	35—40	0
D. The same as A plus 0.01 ml serum II*)	35	0

*) Sera were obtained after incubation of 2 ml 6% human brain suspension with 8 ml human venous blood (serum I) or with 4 ml human serum (serum II) at 37° C for 105 min.

addition of Marcoumar serum, suggesting a similar deficiency in the experimental sera and those of patients treated with Marcoumar. The comparatively long clotting time observed in experiment M indicates that serum obtained from blood treated with brain, in addition to being deficient in factor IX, also lacks another factor required for formation of blood thromboplastin. This may be the Stuart-Prower factor.

In addition, tests were carried out to determine factor VII activity in the experimental sera. Results are given in table 4.

The results show that treatment of blood or serum with brain extract resulted in loss of factor VII activity.

It was of interest to determine which of the tissue components inactivate the serum clotting factors. A number of derivatives of the original brain suspensions

Table 5: Thromboplastin Generation Test Using Sera Obtained after Incubation of Blood or Serum with Different Brain Extracts*)

	Brain Extracts Incubated with:	
	Blood	Serum
	Shortest Clotting Times (sec.)	
A. Brain suspension	46 (23—> 60)	43 (24—62)
B. Brain suspension	15.5	13.5
C. Supernatant of centrifuged brain suspension	35 (12.5—65)	20 (10.5—50)
D. Deposit of centrifuged brain suspension	44 (28—> 60)	48 (36—> 60)
E. Heated brain suspension	> 60	40 (22—> 60)
F. Residue of ether-extracted brain	36 (24—47)	14
G. Residue of ethanol-ether extracted brain	17 (16—17.5)	12
H. Ethanol-ether extract of brain**)	12	10 (9.5—11)
J. NaCl control	11 (9.5—12)	10 (9—11)

*) 8 ml normal human venous blood or 4 ml serum incubated at 37° C for 60—90 min. with brain extracts described below under A—H. Mixtures then centrifuged at 2000 g for 15 min. and supernatant sera used in thromboplastin generation test as described in Fig. 1.

A. 2 ml human brain suspension containing 55 mg organic matter per ml;
B. 0.1 ml human brain suspension as in A;
C. Human brain suspension as in A centrifuged at 2000 g for 30 min.; 2 ml supernatant was used; it contained 12.5 mg organic matter per ml;
D. Deposit from C; in some experiments washed 3 — 10 times with 0.145 M NaCl; resuspended in 0.145 M NaCl to give suspension containing 40 mg organic matter per ml; 2 ml used;
E. Human brain suspension as in A heated in boiling water bath for 5—30 min.; 2 ml used;
F. Dry brain powder extracted 3 times with peroxide-free diethyl ether; residue dried on water bath and suspended in 0.145 M NaCl; 2 ml of suspension used;
G. Dry brain powder extracted 3 times with boiling ethanol-ether (3 : 1); residue dried on water bath and suspended in 0.145 M NaCl; 2 ml of suspension used;
H. Ethanol-ether extract from G evaporated under reduced pressure, suspended in 0.145 M NaCl; 2 ml of suspension used.

**) No further P-lipid addition in thromboplastin generation test.
In experiments D, F, G and H the residues were suspended in 0.145 M NaCl to give a concentration comparable to that in the original brain suspension.

were prepared and these were incubated with blood as well as with serum. The sera obtained were tested for their ability to take part in formation of blood thromboplastin. The results are given in table 5.

The results indicate that the most active components in the inactivation of serum clotting factors are present in the deposit of centrifuged brain suspensions. Such deposits have been washed up to 10 times with 0.145 M NaCl without loss of activity. Heating of brain suspension from 5 to 30 minutes in a boiling water bath did not diminish activity. On the other hand the supernatant of centrifuged brain suspension and the residue of ether-extracted brain showed diminished

Table 6: Thromboplastic Activity of Various Brain Preparations*)

	Clotting Time (sec.)
1. 6% human brain suspension	15 (12—19)
2. 6% human brain suspension centrifuged at 2000 g, 30 min.	15 (11.5—19)
3. Resuspended residue from 2	22 (20.5—22.5)
4. 6% human brain suspension heated at 100° C for 5—30 min.	36 (20.5—52)
5. Resuspended ethanol-ether insoluble residue from human brain	59 (52—67)

*) To 0.1 ml normal human oxalated plasma, 0.2 ml of a mixture of equal volumes of the brain preparation and 0.01 M calcium chloride were added at 37° C. The results are averages, with variations in parentheses.

Table 7: Influence of Rabbit's Brain and Lung on Human Serum Prothromboplastins*)

Composition of Reaction Mixtures	Clotting Time (sec.)	Incubation Time (min.)
1. 2 ml normal human serum + 0.4 ml rabbit's lung suspension	71	4—20
2. 2 ml normal human serum + 0.2 ml rabbit's lung suspension	30	12
3. 2 ml normal human serum + 0.4 ml rabbit's brain suspension	27	18
4. 2 ml normal human serum + 0.2 ml rabbit's brain suspension	15	14
5. 2 ml normal human serum + 0.4 ml NaCl	11	12
6. 0.06 ml serum 3 + 0.03 ml serum 5	14	15
7. 0.06 ml serum 2 + 0.03 ml serum 5	18.5	8
8. 0.03 ml serum 5	13.5	10

*) The organ suspensions were incubated with serum at 37° C for 90 min. and centrifuged at 6° C, 2000 g. 0.06 ml of the supernatant was used serum in a thromboplastin generation test similar to that described in Fig. 1. The times recorded are the shortest clotting times, and the shortest incubation times required for development of maximum activity.

activity. Little or no activity was present both in the insoluble residue of ethanol-ether extracted brain and in the soluble lipid fraction.

Results of determinations of thromboplastic activity of the various brain preparations as indicated by their ability to clot recalcified plasma are given in table 6.

From the results in table 6 it is seen that the supernatant of the centrifuged brain extract was as active as thromboplastin as the original brain suspension. But its capacity to inactivate serum clotting factors was less than that of the original brain suspension. Particularly marked is the difference between thromboplastic and inactivating property in the cases of the residue from the centrifuged suspension and the heated brain suspension. Both had a very low thromboplastic activity but high inactivating potency.

Finally, suspensions of rabbit's brain and lung were prepared and these were incubated with normal human serum. The reaction products were tested for their ability to take part in blood thromboplastin formation. Results are given in table 7.

It is seen that both rabbit's brain and lung inactivate human sera.

Discussion

The presented experiments indicate that organs contain components which inactivate factors VII and IX. It appears that the inactivation does not interfere with blood clotting. Factor IX is not required in the conversion of prothrombin to thrombin by tissue thromboplastins and with regard to factor VII, which is required, the rate of thrombin formation in presence of tissue thromboplastin is perhaps greater than the rate of inactivation of factor VII by the tissue.

The results suggest that a heat stable lipid or lipoprotein is involved in the inactivation. This process is not related to tissue thromboplastin activity. It is perhaps due to an adsorption of the clotting factors on the tissue component. In this connection it should be pointed out that factor V and factor VII can be adsorbed by thromboplastic compounds (17) and the product activates prothrombin conversion. On the other hand several workers (18—23) have described clotting inhibitors of a lipid nature and it is possible that some of these materials are responsible for the observed inactivation of the clotting factors reported here.

The tissue component which inactivates factor IX and factor VII may play some role in thrombotic phenomena. As an antagonist to these clotting factors, the action would be analogous to antithrombins which remove an excess of thrombin from clotting blood and thus prevent a thrombus from spreading.

Summary

Brain and lung suspensions incubated with blood or serum produce sera which are deficient in factor IX and factor VII.

Suspensions prepared from acetone-dehydrated brain are effective substitutes for free phospholipids in the formation of blood thromboplastin.

The fraction which inactivates the clotting factors is connected with a readily sedimentable portion of the brain suspension.

It is heat stable, and destroyed by ethanol-ether extraction.

The extracted lipid is inactive.

Résumé

L'incubation d'émulsions de cerveau ou de poumons avec du sang ou du sérum donne lieu à la formation de sérum dépourvu des facteurs VII et IX.

Les suspensions de cerveau déhydraté à l'acétone sont des substitutifs adéquats pour les phospholipides libres qui interviennent dans la génération de la thromboplastine.

La fraction du cerveau qui inactive les facteurs de la coagulation est en rapport avec des particules facilement sédimentables, résiste à la température et est détruite par le mélange d'éthanol et d'éther. La lipide extrahée est inactif.

Zusammenfassung

Werden Hirn- und Lungensuspensionen mit Blut oder Serum inkubiert, so entstehen Sera, die keinen Faktor IX und VII enthalten.

Suspensionen von azeton-getrockneter Hirnsubstanz ersetzen das freie Phospholipid bei der Thrombokinasebildung wirksam.

Die Fraktion, die die Gerinnungsfaktoren inaktiviert, findet sich in einem leicht sedimentierbaren Anteil der Hirnsuspension.

Sie ist hitzestabil und wird durch Äthyläther extrahiert. Das extrahierte Lipid ist inaktiv.

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