The Mandatory Role of Lipid in the Interaction of Factors VIII and IX.* (31590)

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Two groups have provided kinetic data which strongly support the hypothesis that activated Factor IX (IX_a, activated PTC) catalyzes the activation of Factor VIII (AHG)(1,2), but the cofactors in the two systems differ. Both groups used reaction mixtures containing sources of Factor VIII, Factor IX_a, and calcium. To these one group added thrombin(2) and found no other cofactor requirement. The other group did not add thrombin, and their reaction mixture required lipid to obtain full activation of Factor VIII(1). It seemed probable that the two reaction mixtures ultimately included the same reactants, either generated in the mixture or added as contaminants. Minute amounts of thrombin increase Factor VIII activity, and this alteration appears to be a prerequisite for Factor VIII to interact with the clotting factors in serum(3,4,5). Lipid is known to be required in a late step in clotting leading to the generation of thrombin(6). Therefore, one possible explanation for the different cofactor requirements of the two reaction mixtures could be that lipid was required in the mixture without added thrombin not for the Factor IX_a-VIII interaction, but to generate a trace of thrombin in the reaction mixture. This thrombin would then participate directly in the activation of Factor VIII. Alternatively, lipid might act directly in the Factor IX_a-VIII interaction, and the reaction mixture of the group who added only thrombin might have been contaminated with sufficient lipid to satisfy this requirement.

To test whether lipid participated directly in the interaction of Factors IX_a and VIII, we prepared sources of Factors VIII and IX_a which contained no significant lipid. These were allowed to react in the presence or absence of added lipid with calcium and a concentration of thrombin far in excess of that potentially available in the reactants. Under these conditions the generation of small amounts of thrombin in the reaction mixture would have no effect, and if lipid were still required for activation of Factor VIII, it would have to be a true cofactor of the reaction.

Materials. Factor VIII source was citrated hereditary Factor V deficient plasma which was adsorbed twice with $Al(OH)_3$ (Cutter Laboratories, Berkeley, Calif.) to remove Factors II, VII, IX and X, and then centrifuged in a Spinco Model L ultracentrifuge at 40,000 rpm for 60 minutes to remove platelet lipid. The center layer which had very small amounts of Platelet Factor 3 activity was carefully separated and frozen. (Clotting times in the Platelet Factor 3 assay: uncentrifuged—about 60″, center layer—92″, blank—97″.) Clotting factor activities of this reagent are shown in Table I. *Normal serum.* Blood was drawn into 16 × 100 mm glass tubes and incubated at 37°C for 15 minutes after clotting, the clots were removed by centrifugation, and 20 mg of diatomaceous earth powder, Dicalite 4200 (Great Lakes Carbon Corp., Los Angeles, Calif.) were added for each ml of supernatant serum. After 2 hours incubation at 37°C, most of the powder was removed by centrifugation for 10 minutes in a clinical centrifuge, and the serum was decalcified by adding 0.2 ml anticoagulant to each 1 ml of serum. To reduce lipid the anticoagulated serum was centrifuged for 60 minutes at 40,000 rpm in the ultracentrifuge, and the middle third of the serum was carefully removed and frozen in small aliquots. (Clotting times in the Platelet Factor 3 assay: uncentrifuged—about 26″, center layer—75″, blank—83″.) Clotting factor activities of this reagent are shown in Table I. The Factor IX assay does not distinguish between native and activated Factor IX, but the treatment of the serum

* Supported by grant H-06128-06 from National Heart Inst., USPHS.

TABLE I. Clotting Factor Activities of Reactants.

Reagent	Activity (%)* II	V	VIII	IX	X	Platelet Factor 3 (platelet equiv/mm³)
Adsorbed Factor V deficient plasma	.5	.2	43	.2	0	75
Normal serum	.9	3.8	14.5†	108	152	41
Factor X deficient serum	32	4.5	1	96	.3	9,100
Factor IX deficient serum	54	—	13 †	2	144	26,000

* 100% is the activity in a normal plasma pool.
† These relatively high activities presumably represent an effect of active intermediates beyond Factor VIII which influence the assay.

with diatomaceous earth powder should have converted most of the Factor IX to the activated form(7). *Factor X deficient serum.* Blood from a patient with a hereditary deficiency of Factor X was clotted in a glass tube, incubated at 37°C for 2 hours, anticoagulated in a ratio of 1 part anticoagulant to 9 parts blood, centrifuged to remove the clot, and lyophylized. It was later reconstituted and stored in small frozen aliquots. Clotting activities are shown in Table I. *Factor IX deficient serum.* Blood from a patient with a hereditary deficiency of Factor IX was incubated in glass tubes at 37°C for 2 hours after clotting, centrifuged to remove the clots, anticoagulated in a ratio of 1 part anticoagulant to 5 parts serum, and frozen in small aliquots. Clotting factor activities are shown in Table I. Lipid source was cephalin, an acetone insoluble, ether soluble extract of human brain prepared as described previously (8). Undiluted cephalin had a Platelet Factor 3 activity equivalent to plasma containing 1.34×10^7 platelets/mm³. *Substrate plasma* was plasma from a patient with severe hemophilia A. *Anticoagulant* is a solution of 0.06 M sodium citrate and 0.04 M citric acid. *Thrombin.* Purified human thrombin, prepared as described elsewhere, was supplied by Dr. Kent Miller of Albany, New York(9).

Methods. Clotting factor assays. Detailed conditions for determination of Factors VIII (AHG)(7), IX (contact PTC assay)(10), and V (proaccelerin)(11) have been described elsewhere. Factor II (prothrombin) was measured by a one-stage venom cephalin technique(8) to which a serum reagent was added to supply Factor X. Factor X (Stuart factor) was measured by a slight modification of Hougie's technique(12). Thrombin concentrations were determined as described previously(13). Platelet Factor 3 activity was measured in a one-stage assay. The substrate was a platelet poor plasma prepared according to Husom(14) and stored at —20°C. The standard was platelet rich plasma containing 414,000 platelets/mm³ which had been frozen and thawed twice to break the platelets, then refrozen in small aliquots until tested. The test system consisted of 0.1 ml substrate, 0.1 ml diluted standard or test substance, and 0.1 ml Russell's viper venom ("Stypven," Wellcome Research Laboratories, Beckenham, England), 1/160,000 in barbital buffer, incubated for 3 minutes at 37°C, followed by 0.1 ml 35 mM calcium chloride. The clotting times of test samples were converted to equivalents of broken platelets in plasma from a curve made from dilutions of the standard platelet rich plasma by plotting log clotting time *versus* log plasma concentration. *Determination of experimental reaction.* Reaction mixtures were prepared by incubating diluted adsorbed Factor V deficient plasma, a cephalin-calcium reagent, and thrombin in a plastic tube at 37°C. Thirty seconds later a serum reagent was added, and this was noted as zero time of the reaction. In control experiments buffer was substituted for the cephalin and saline for the calcium, and the substrate concentrations of cephalin, calcium, or both were raised so that the over-all concentrations in the clotting mixture remained constant. Details are given in the footnotes to the table.

The substrates consisted of 0.1 ml Factor VIII deficient plasma and 0.1 ml cephalin 1/100 (or 1/80 when no cephalin was added to the reaction mixture) in a plastic tube.

TABLE II. Effect of Sera Deficient in Factor X or Factor IX in Mixtures of Factor VIII, Thrombin, Lipid, and Calcium.*

Serum reagent	Substrate clotting time (sec) Incubation time 0	2 min	3 min
1. Factor X deficient serum 1/20	144	119	127†
2. Factor IX deficient serum 1/20	155	159	180†
3. Factor X deficient serum 1/10 + Factor IX deficient serum 1/10	138	45	44
4. Buffer	190	201	229

* Reaction mixtures contained 0.1 ml adsorbed Factor V deficient plasma 1/4, 0.1 ml of a mixture containing cephalin 1/100 and calcium 40 mM, and 0.1 ml thrombin 0.5 NIH u/ml. Thirty seconds later 0.1 ml of serum was added and then at intervals shown 0.1 ml of the complete mixture was added to the substrate (see *Methods*).

† Average of 4 experiments.

These were incubated for 3 minutes at 37°C; then 0.1 ml of 20 mM calcium chloride (or 30 mM when saline was used in the test mixture) was added followed by 0.1 ml of reaction mixture, and the clotting time noted.

Results. 1. *Evidence that a reaction takes place between Factors VIII and IX in presence of thrombin, lipid, and calcium.* The results of incubating sources of Factor VIII (adsorbed Factor V deficient plasma) and activated Factor IX (Factor X deficient serum) in the presence of all proposed cofactors is shown in Table II, line 1. A product forms briefly but highly reproducibly which results in a small change in substrate clotting time. If Factor IX deficient serum is substituted, no reaction occurs, line 2. If the 2 sera are combined so that Factors IX and X are both available, a powerful and more stable clotting activity is generated, line 3. 2. *Evidence that lipid is required for a reaction in presence of thrombin.* In these experiments normal serum, a more readily available source of activated Factor IX, was substituted for Factor X deficient serum. The results of a typical experiment in which we incubated Factor VIII, thrombin, calcium, lipid, and serum are shown in Table III along with suitable controls. The complete reaction mixture generated a powerful clotting activity in 2 minutes, as shown in line 1. If lipid, calcium, serum, or Factor VIII were removed from the mixture, no time consuming reaction took place, lines 2 through 6. Of particular importance are the data in line 2 which clearly show that without the lipid no reaction occurs. The reaction between Factor VIII and serum factors in the presence of excess thrombin *requires* lipid.

Discussion and conclusions. It is established that lipid is required at a late stage in intrinsic clotting in which Factor X reacts with Factor V to form the activity which catalyzes the conversion of prothrombin to thrombin(15). Recently, it has been proposed that lipid is also required for an earlier reaction in which Factor VIII is activated(1). For reasons outlined in the introduction we

TABLE III. Effect of Lipid on a Reaction Between Factor VIII, Serum Factors, Thrombin, and Calcium.

	Components of reaction mixture* Factor VIII (adsorbed Factor V deficient plasma)	Normal serum	Calcium	Thrombin†	Lipid	Substrate clotting time (sec) Incubation time 0	2 min	3 min
1.	+	+	+	+	+	58	22	24
2.	+	+	+	+	—	87	102	104
3.	+	+	—	+	—	92	114	115
4.	+	+	—	+	+	80	106	100
5.	+	—	+	+	+	109	145	203
6.	—	+	+	+	+	107	129	127

* Complete reaction mixture contained 0.1 ml adsorbed Factor V deficient plasma 1/4, 0.1 ml of a mixture containing cephalin 1/100 and calcium 40 mM, and 0.1 ml thrombin 1 NIH u/ml preincubated for 30″ at 37°C, followed by 0.1 ml serum 1/20. Buffer or saline was substituted for the different reactants in control experiments. At the times shown above, 0.1 ml of the reaction mixture was added to a warmed substrate mixture (see *Methods*).

† This concentration greatly exceeds the potential thrombin of the reactants.

felt it was important to determine whether a lipid dependent reaction between Factor VIII and activated Factor IX could still be demonstrated in the presence of excess thrombin.

The initial experiments shown above confirm that Factor VIII activation in a reaction mixture containing lipid, calcium, and thrombin requires Factor IX and is independent of Factor X. The reaction product which forms in the absence of Factor X, presumably activated Factor VIII, is very labile, and only a small degree of activation can be detected at any time. Macfarlane *et al*(2) also report that activated Factor VIII is unstable, though our activity appears to decay more rapidly, perhaps because of the higher concentration of thrombin in our reaction mixtures. It is important to emphasize that because activated Factor VIII is labile and produces only small changes in substrate clotting times, it may easily escape detection if Factor X is absent. In the presence of Factor X the reaction sequence continues, and activated Factor X is formed(6). This clots the substrate very rapidly and is therefore readily measured.

For this reason the requirement for a lipid cofactor in the presence of thrombin was studied using normal serum which contains both Factors IX and X. The results show that, if reagents are carefully prepared to contain minimal lipid contamination, no detectable reaction will occur between serum and a Factor VIII reagent preincubated with thrombin and calcium unless exogenous lipid is added. This means that lipid must participate directly in an early reaction between the serum factors and Factor VIII.

We have not quantitated the amount of lipid required for the activation of Factor VIII. However, this must be very small because the lipid present as a contaminant in the diluted normal adsorbed plasma and serum reagents of the "Product I test"(16) will allow a reaction to occur. For example, with standard diluted reagents for this test, containing lipid activity equivalent to about 350 platelets per mm^3, the substrate clotting time shortened from 55″ at 0 minute to 27″ after 25 minutes. The same reagents, with lipid activity reduced by ultracentrifugation to the equivalent of 12 platelets per mm^3, were almost inactive in the test, giving a substrate clotting time of 52″ at 0 minute and 45″ after 25 minutes.

This dramatic effect of traces of lipid accounts for the apparent conflict between our data and older literature which showed that exogenous lipid was not required for an interaction between adsorbed plasma, serum, and calcium(16). The amount of lipid required for activation is so small that it will be present as a contaminant in reagents prepared by older standard techniques. Only with reagents specially prepared to reduce lipid contamination can the mandatory role of lipid in the activation of Factor VIII be demonstrated.

Summary. 1. An interaction between activated Factor IX and Factor VIII which is independent of Factor X has been confirmed using human reagents. The product, activated Factor VIII, is a very labile activity. 2. Lipid has been shown to be a mandatory cofactor in the interaction of Factor VIII and serum factors in the presence of thrombin.

We wish to thank Dr. Kent Miller for supplying us with purified human thrombin and Miss Lorraine Gonyea for supplying us with hereditary Factor X deficient serum.

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Received July 5, 1966. P.S.E.B.M., 1966, v123.

Immunologic and Chemotherapeutic Effects on Human Melanoma Heterotransplants.*† (31591)

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Lymphocytes have been known to inhibit or restrain tumor and other tissue transplants since the experiments of Murphy in 1926(1). The sequential transplantation of human cancers in hamster cheek pouches usually requires preconditioning by cortisone or irradiation, which are thought to repress lymphocyte-borne immune responses. In the present experiments human melanoma (ME-1) transplants grew larger in cortisone preconditioned hamsters that were treated with antilymphocyte serum. Conversely, immunologically enhanced rejection of tumors was achieved by pretreating hamsters with footpad injections of frozen tumor and Freund's adjuvant.

Methotrexate treatment significantly reduces the growth of the ME-1 tumor(2). The therapeutic effect persisted when treated tumors were subsequently transplanted into untreated hamsters.

Materials and methods. A human malignant melanoma (ME-1) previously studied in other investigations was transplanted, utilizing the same technique, size of male hamsters, diet and amount of cortisone as already reported(2,3).

To prepare antilymphocyte sera, the axillary, cervical and mesenteric lymph nodes of normal hamsters were mechanically minced, suspended in physiologic saline solution, and the mixture filtered through wire mesh. The washed lymphocytes were mixed with equal volumes of incomplete Freund's adjuvant, without a bacterial component. From 20,000 to 100,000 lymphocytes were present per ml of saline. The antigen was administered to rabbits, 1.5 ml subcutaneously, once a week for 4 injections. The animals were bled for antiserum 7 to 10 days after the last injection.

Antilymphocyte activity of the rabbit antisera was tested by injecting 1 ml intraperitoneally into normal hamsters. Single injections reduced the blood lymphocytes and monocytes from an average of 65% to an average of 33% for a period of 6 days thereafter. Attempts to quantitate the changes further by lymphocyte counts were unsuccessful, due to large variations in numbers of blood lymphocytes in both control and antiserum-injected hamsters. Tests of the antisera by precipitin and complement-fixation methods, using lysed lymphocytes as antigen, showed no reactions.

One intraperitoneal injection of 1 ml rabbit antiserum was given to 25 tumor-bearing hamsters on the fourth day after tumor implantation, in order to produce a maximal effect at the beginning of the logarithmic (log) phase of tumor growth(2).

Methotrexate was administered as previously reported, 10 mg intraperitoneally, to transplant donors, from which tumors were removed after 21 days of growth. These harvested tumors were implanted into 13 hamsters preconditioned with cortisone as usual, but not otherwise treated.

* Aided by USPHS Grants: CY-6330 and AM-06578-01.

† Reprint 172 of Scripps Clinic and Research Foundation.