Tissue Factor Activity in Lymphocyte Cultures from Normal Individuals and Patients with Hemophilia A

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ABSTRACT The procoagulant material of lymphocytes has been characterized as tissue factor. Lymphocytes stimulated with phytohemagglutinin or the purified protein derivative of the tubercle bacillus developed procoagulant activity with incubation in tissue culture. While this material corrected the prolonged clotting time of factor VIII (AHF) deficient plasma, we have shown, utilizing a sensitive radioimmunoassay, that no AHF antigen was present in the cell cultures. Further, we have demonstrated this material to be tissue factor by coagulation techniques and immunological cross-reactivity. The published data regarding factor VIII synthesis is reviewed in light of these observations and comments are made regarding the role of the lymphocyte procoagulant.

INTRODUCTION

The leukocyte has been implicated as a source of antihemophilic factor (AHF—factor VIII)[1] by several investigators (1, 2). It has been known since 1911 that both normal and leukemic white cells contain material that enhanced in vitro clotting (3–14), and recent studies have demonstrated this material to be tissue factor (15–19). Nevertheless, reports of elevated factor VIII levels in blood from patients with leukocytosis (20–22) and of enhanced factor VIII levels in blood from a hemophilic child with acute lymphoblastic leukemia have been used as evidence that factor VIII is synthesized by white blood cells (2).

In addition, the results of splenic perfusion studies (23–26), cell infusions (27), and splenic transplantation in both animals and humans (28–30) were used to support the hypothesis that the spleen either stores, produces, or participates in the production of factor VIII. Since the spleen is predominantly a lymphoid organ, it seemed possible that lymphocytes played an important role in the increased plasma levels of factor VIII following splenic transplantation.

The conflicting data of some perfusion experiments (31) and the lack of more specific assays for both tissue factor and factor VIII have limited the interpretation of these earlier studies because the material assayed as factor VIII activity might have been tissue factor.

The purpose of this paper is to show that stimulated lymphocytes obtained from both normal individuals and patients with hemophilia A (classic hemophilia) contained tissue factor activity and that despite significant correction of factor VIII-deficient plasma in a coagulation assay no antihemophilic factor (AHF) antigen was demonstrated using a sensitive radioimmunoassay technique.

METHODS

Lymphocyte cultures. Whole blood from normal subjects and patients with hemophilia A was collected in 50-ml portions by careful venipuncture, utilizing plastic syringes containing 1 ml sodium heparin (1,000 U.S.P. U/ml, Up-

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[1] *Abbreviations used in this paper:* AHF, antihemophilic factor; CNP, control normal plasma; IBS, imadazole-saline buffer; PHA, phytohemagglutinin; PPD, purified protein derivative of tubercle bacillus; PTT, partial thromboplastin time; SBB, saline-barbital buffer.

john Co., Kalamazoo, Mich.) and 5 ml of 6% Dextran solution in 5% dextrose in water (Pharmachem Corp., Bethlehem, Pa.). The lymphocytes were obtained by a modification of the method of Boyum (32). The blood was sedimented upright in the syringes for 1 h at 37°C. Leukocyte-rich supernatant was then expressed into a 50 ml graduated Teflon culture tube (Falcon Plastics, Oxnard, Calif.). Centrifugation was carried out at 200 *g* at room temperature for 10 min. The supernatant plasma was discarded, and the cell button was resuspended in 3.5 ml of TC-199 tissue culture media (Grand Island Biological Co., Grand Island, N. Y.). The cell button was agitated gently on a Super Mixer (Matheson Scientific, Kansas City, Mo.) to ensure adequate dispersion. A Ficoll-Hypaque density gradient was prepared as follows: 9.556 g Ficoll (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) was mixed with 30.0 ml of a 50% sodium Hypaque solution (Winthrop Laboratories, Sterling Drug, Inc., New York) and 120.4 ml of distilled water. The solution was obtained by stirring the mixture on a heating plate and was then filtered through a 0.4 μm Millipore filter (Millipore Corp., Bedford, Mass.), and 4-ml portions were stored at 4°C until use.

The resuspended cell button was then gently layered onto the 4 ml Ficoll-Hypaque gradient in a 16 × 125 mm plastic culture tube (Falcon Plastics). All materials were handled under sterile conditions. The gradient was then centrifuged at 370 *g* for 30 min at room temperature.

The mononuclear cell layer from the top of the gradient, containing lymphocytes and monocytes, and the granulocyte layer from the bottom of the gradient were gently aspirated and transferred to respective 50-ml conical, graduated culture tubes. The volumes were adjusted to 20 ml each with TC-199 media, and the cell count and differential were determined. Cell viability was determined by the exclusion of trypan blue dye at the initiation and termination of the incubation periods (33). Cultures were discarded or applied to a new gradient if less than 96% mononuclear cells were present or less than 85% of the cells failed to exclude the trypan blue dye. The cells were then resedimented by centrifugation at 200 *g* for 10 min at room temperature, and the supernatant was discarded. Penicillin and streptomycin (Microbiological Associates, Inc., Bethesda, Md.) were added to fresh media at a concentration of 0.5% by volume, and glutamine (Microbiological Associates) was added at 1% concentration. No additional serum or nutrients were utilized.

The cells were then resuspended to a concentration of 1.0×10^6 cells/ml with the enriched media and transferred to 12 × 75 mm polystyrene plastic culture tubes (Falcon Plastics) for incubation. Phytohemagglutinin (PHA, Burroughs Wellcome & Co., Research Triangle Park, N. C.) was used as a mitogen at 1 μg/culture. A purified protein derivative of the tubercle bacillus (PPD, Connaught Medical Research Laboratories, University of Toronto, Toronto, Canada) was used as an antigen at a concentration of 2 mg protein/ml (10 μl/culture). PHA and PPD were added to both lymphocytes and granulocyte cultures. After variable periods of incubation at 37° in a 5% CO_2 media, the cells were either quick frozen and stored at −70°C until the time of assay or immediately subjected to ultrasonic disruption for 45 s at 4°C (Biosonic, Bronwell Scientific, Inc., Rochester, N. Y.; probe at 60). Ultrasonic disruption of the cell cultures was carried out in their original media (1.0×10^6 cells/ml) following thorough agitation to resuspend the cell buttons. Coagulation studies were performed immediately following disruption without prior centrifugation. Parallel cell cultures, used to determine response to mitogen and antigen, were reincubated with [^{3}H]thymidine (New England Nuclear Corp., Boston, Mass; 6.7 μCi/ml) for 4 h and harvested over 0.45-μm Millipore filters by the method of Hartzman, Segall, Bach, and Bach (34). All cultures were harvested in triplicate (e.g., three stimulated and three controls for each experiment). Nucleoprotein was precipitated onto the filters with perchloric acid, and the filters were washed twice with ethyl alcohol. They were then dried in an oven at 80°C for 45 min and placed in scintillation fluid (42 ml Liquifluor [New England Nuclear] per 1,000 ml toluene). Counts per minute were measured in a Beckman Model LS150 liquid scintillation system (Beckman Instruments, Inc., Silver Spring, Md.). Significant uptake of the isotope, indicating stimulation of the cells, was defined as:

$$\frac{\text{cpm-stimulated cells}}{\text{cpm-control cells}} > 3.0.$$

Coagulation tests. The test for procoagulant activity was a modification of the unactivated partial thromboplastin time (PTT) of Langdell, Wagner, and Brinkhous (35), utilizing Platelin (Warner-Chilcott Laboratories, Morris Plains, N. J.) as a source of brain cephalin. In this system 0.1 ml control normal plasma (Dade CNP, Dade Division, American Hospital Supply Corp., Miami, Fla.) and either 0.1 ml of saline-barbital buffer or 0.1 ml of the sonicated, lymphocyte suspension were incubated together for 2 min at 37°. Platelin (0.1 ml) and 0.1 of 0.025 M calcium chloride were then added, and the clotting time was recorded. Prothrombin time, utilizing Ortho Brain Thromboplastin (Ortho Pharmaceutical Corp., Raritan, N. J.), was performed by the method of Quick (36), and assays for factors II, V, VII, and X were performed in a prothrombin time system. Factor II-deficient reagent was prepared by a modification of the method of Pechet (37). The reagent was prepared from citrated rather than oxalated serum (9 vol of human serum and 1 vol of sodium citrate, 3.8%). The only other modification of the original method utilized aluminum hydroxide (Amphojel, Wyeth Laboratories, Inc., Philadelphia, Pa.), 0.1 ml per 1.0 ml of citrated plasma, in place of barium sulfate. Factor V-deficient and factor X-deficient reagents were obtained from patients with the respective deficiency states. Factor VII-X complex reagent was prepared by the method of Bachmann, Duckert, and Koller (38). Levels of factors VIII, IX, XI, and XII were determined in an activated partial thromboplastin assay system, modified from the method of Proctor and Rapaport (39), utilizing Platelin plus Activator (Warner-Chilcott Laboratories). The lymphocyte suspensions (0.1 ml) and 0.1 ml of either a 1:10 dilution of CNP or saline-barbital buffer was preincubated with 0.1 ml of the respective factor-deficient substrate for 2 min at 37°C. Platein plus Activator (0.1 ml) was then added, and the reagents were allowed to incubate for a further 5 min at 37°C. Calcium chloride (0.1 ml) was then added, and the clotting time was measured. All deficient substrates for the activated partial thromboplastin assay system were obtained from patients with the respective deficiency states. The presence of thrombin was evaluated by a modification of the method of Hardisty and Ingram (40) utilizing semipurified fibrinogen (Warner-Chilcott Laboratories); 0.2 ml of the fibrinogen was clotted with either 0.1 ml of the sample to be assayed or 0.1 ml of human thrombin (Ortho Pharmaceutical Corp.) as a standard at a concentration of 6.25 NIH U/ml of diluent.

The calcium chloride concentration used throughout the experiments was 0.025 M. Imidazole-saline buffer (IBS)

for the tissue factor experiments was made with 0.05 M imidazole and 0.10 M sodium chloride and adjusted to pH 7.3 with concentrated hydrochloric acid. Saline-barbital buffer (SBB), used as diluent for all other coagulation tests, was prepared with 0.1 M sodium barbital, 0.15 M barbital, and 0.125 M sodium chloride and adjusted to pH 7.34. Control normal plasma was reconstituted fresh each day from 1.0 ml portions of a lyophilized pool (Dade CNP).

The procoagulant assays were performed on a Fibrometer (BioQuest Div., Becton, Dickinson & Co., Cockeysville, Md.) as were the prothrombin complex assays. The intrinsic system factors were assayed on a Clotek instrument (Hyland Div., Travenol Laboratories, Inc., Costa Mesa, Calif.).

Tissue factor assay. Tissue factor was assayed by the two-stage method described by Nemerson (41), using bovine serum eluate as a source for factors VII and X and bovine plasma as the substrate for activated factor X. Tissue factor activity of the various cultures was compared with the activity of the bovine brain standard used by Nemerson (41).

Tissue factor antibody. Human placental tissue factor prepared by the method of Nemerson and Pitlick (42) was used to immunize New Zealand albino rabbits as described by Zeldis, Nemerson, Pitlick, and Lentz (43). In brief, human placenta was homogenized and dehydrated by washing with large volumes of acetone followed by air-drying. The acetone powders were extracted with heptane-butanol (2:1, 20 ml/g) to remove phospholipids and then extracted with 0.5 M NaCl to remove extraneous proteins. Following solubilization with 0.25% sodium deoxycholate (Mann Research Labs, New York), the soluble material was brought to 30% saturation with solid ammonium sulfate and filtered, and the precipitate was discarded. The filtrate was brought to 60% saturation with solid ammonium sulfate, and the precipitate was collected by centrifugation. The apoprotein was then resuspended and dialyzed against imidazole-saline buffer (0.05 M imidazole, 0.375 M NaCl, pH 7.2) at 4°C. The dialyzed material was concentrated by filtration (Diaflo XM-50 filter) and then gel filtered on Sepharose 6B (Pharmacia). Active fractions, as determined by the assay previously described (41), were pooled and rechromatographed on Sepharose 6B. Criteria for purity are detailed in the original reference (42). The placental tissue factor had a specific activity of 10,000 U of tissue factor/mg protein. The rabbits were given an initial intradermal injection of 1 mg of the purified human tissue factor with complete Freund's adjuvant and subsequent biweekly injections of 100 μg of the antigen. Antiserum was harvested 2 wk after the third injection and then 2 wk after subsequent injections. The rabbit antibody neutralized tissue factor from human brain and placenta. Two precipitin lines appeared on immunodiffusion plates; these probably correspond to the two species previously noted (42). The rabbit antibody neutralized coagulant activity of human brain acetone powders or purified placental tissue factor. Before use in the neutralization assays, both normal rabbit serum and rabbit antiserum to human tissue factor were absorbed with rabbit lung acetone powder (5 mg/ml), barium sulfate (100 mg/ml, three times), heated at 60°C for 30 min, then precipitated with $\frac{1}{3}$ vol of saturated ammonium sulfate. The precipitate was resuspended in a volume of water equal to the starting volume and dialyzed overnight in IBS.

Inhibition of tissue factor activity. An acetone powder of human brain was prepared by the method of Nemerson (41). This preparation was then homogenized and sonicated 15 s in IBS. This suspension was diluted until it was equivalent to 100 U of bovine tissue factor in the two-stage coagulation assay. The semipurified rabbit globulin described above was then serially diluted and incubated with the human brain thromboplastin at 37°C for 30 min. For studies of inhibition of coagulant activity developed by the cultures, the globulin was used at that dilution which just inhibited all of the brain tissue factor. Globulin from the nonimmune rabbits served as a control for all experiments. The antigen and antibody were incubated in equal volumes for all determinations and compared with an equivalent dilution of the human brain tissue factor.

AHF antigen determination. AHF antigen was measured using a radioimmunoassay described in detail by Hoyer (44). Rabbit anti-human AHF prepared by a minor modification of the method of Zimmerman, Ratnoff, and Powell (45) was purified and radiolabeled for use in this assay (46). The ^{125}I-labeled IgG anti-AHF (0.5 ml) was incubated with 5 ml normal human plasma, and soluble antigen-antibody complexes were separated by gel filtration of the mixture using Sepharose 6B (1.6 × 60 cm, 25°C, flow rate of 20 ml/h, saline-barbital buffer). These complexes (void volume fractions) were then dissociated by dialysis using glycine-saline buffer, pH 2.4, prior to gel filtration on Sephadex G-200 (1.5 × 90 cm, 25°C, flow rate of 10 ml/h) equilibrated with the same buffer. The purified ^{125}I-labeled rabbit IgG was then dialyzed against SBB.

Material tested for AHF antigen was incubated with the anti-AHF, and bound, radiolabeled antibody was separated from free antibody by its insolubility in 25% saturated ammonium sulfate. The precipitated radioactivity was proportional to the amount of AHF antigen added, and a standard curve was prepared by plotting the percentage of ^{125}I-anti-AHF counts precipitated (bound/total) vs. log microliters normal plasma. The least squares regression line for the normal plasma standard was used to calculate AHF antigen content for at least two dilutions of each test sample. The standard for antigen measurements was a plasma pool from 20 normal individuals stored at −70°C for less than 2 mo. The antibody-binding capacity of this pool was defined as being 100 antigen units/100 ml.

RESULTS

Lymphocytes obtained from normal individuals and harvested after 27 h of stimulation by either PHA or PPD generated significantly greater procoagulant activity than control (unstimulated) cells in the one-stage factor VIII assay (Fig. 1). 35 control cultures seldom shortened the substrate clotting time. Cells from the same individuals, stimulated either with PHA or PPD, consistently demonstrated shortening of the factor VIII-deficient substrate clotting time by an average of 43 and 33, respectively. Similar results were obtained when cultures were evaluated from five hemophiliacs. Stimulated cells consistently exceeded control cells in the production of the procoagulant activity at the same cell concentration for all experiments described (Fig. 1). There was no statistically significant difference between the shortening of the substrate blank time by the lymphocytes from the hemophiliacs and the cells from the normal donors ($P > 0.30$).

In spite of these data for lymphocytes, the previous association of factor VIII with these cells required further

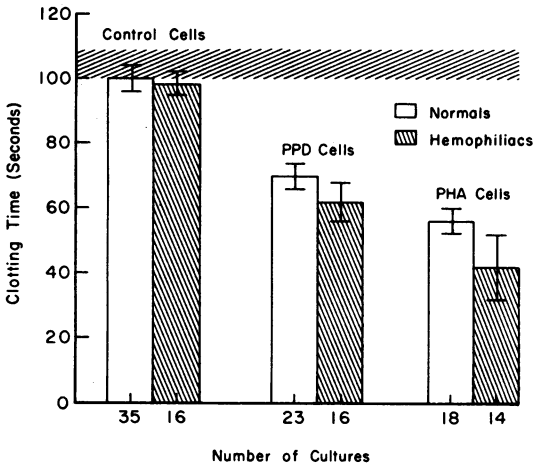

FIGURE 1 One-stage factor VIII assays. Each bar represents the mean clotting time (±1 SEM) of the respective group of cell cultures in the celite-activated factor VIII assay procedure. The bar at the top of the graph represents the clotting time of the VIII-deficient substrate with buffer alone (±1 SEM). All cultures for this experiment were harvested at 72 h and contained 1.0×10^6 cells. Assays were performed on these individual cultures as described in the methods section.

study of the possibility that the procoagulant material was related to factor VIII. Parallel cultures previously assayed for clotting activity and subsequently frozen at −70°C were evaluated for AHF antigen content and compared with levels of antigen in normal plasmas, plasmas from patients with hemophilia A, and plasmas from patients with von Willebrand's disease. None of the cultures of stimulated lymphocytes from either hemophilic patients or from normal individuals significantly exceeded the buffer control in the radioimmunoassay (Fig. 2). AHF antigen determinations in 24 normal individuals revealed a mean level of 108±7 U (1 SEM)/100 ml as compared with the 25 hemophiliacs (156±9) and the 11 patients with von Willebrand's disease (16±3.5). Separate experiments utilized cell concentrations of 8×10^6/ml with no significant activity in the immunoassay for AHF antigen.

The cellular material was then evaluated in other one-stage assay systems against deficient substrates (Table I). The stimulated lymphocytes from either normal subjects or patients with hemophilia corrected substrates deficient in factor XII, XI, IX, and VIII, as well as or better than a standard 1:10 dilution of control normal plasma. No significant correction was obtained with substrates deficient in either factors II, VII–X, or V, and the lymphocytes failed to clot a semipurified preparation of fibrinogen.

These data suggested that the lymphocyte procoagulant acted between factor VIII and factor X in the clotting cascade and led to an investigation of tissue factor. Using a previously described method (41), tissue factor was indeed demonstrated in the stimulated cells (Fig. 3). This two-stage assay procedure has been shown to be

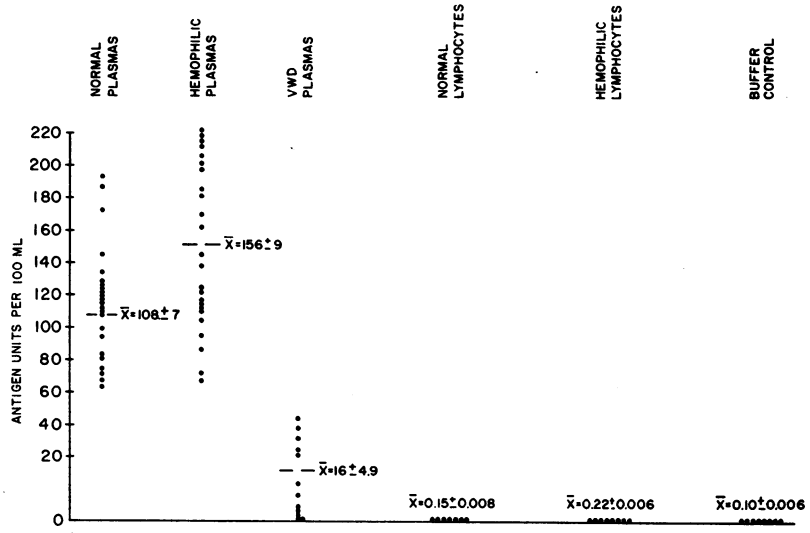

FIGURE 2 Radioimmunoassay for AHF antigen. Each point on this distribution graph represents the antigen determination for an individual donor or a tissue culture sample, and the means are illustrated for each group (±1 SEM). The columns from left to right include: 24 normal plasmas, 25 hemophilic plasmas, 11 von Willebrand (VWD) plasmas, 48 stimulated lymphocyte cultures from normals, 21 stimulated lymphocyte cultures from hemophiliacs, and 50 buffer controls. 1 U of AHF antigen is defined as that present in 1 ml of pooled normal plasma. All cultures for this experiment were harvested at 72 h.

TABLE I

*Correction of Deficient Substrates in One-Stage Assay Systems**

Clotting factor	Buffer time	Normal plasma (1:10 dilution)	Lymphocytes
	s±1 SEM	*s±1 SEM*	*s±1 SEM*
XII	171.2±0.4	65.9±1.4	42.1±3.3
XI	112.0±4.0	69.9±0.3	45.1±0.5
IX	100.7±4.1	56.3±0.3	39.4±11.7
VIII	104.8±0.7	59.8±0.3	39.2±1.7
VII–X	84.9±0.05	21.9±1.6	96.5±2.9
X	181.5±3.0	21.5±0.5	149.9±10.0
II	61.1±0.1	20.1±0.3	65.8±7.4
V	60.2±0.8	25.0±0.3	88.4±0.5
Clotting time of fibrinogen‡	300	12.5±0.3§	300.0

* Each value represents the mean of five or more determinations.

‡ Warner-Chilcott Laboratories.

§ Human thrombin (Fibrindex, Ortho Diagnostics, 6.25 NIH U/ml substituted for normal plasma).

specific for tissue factor (41). PPD- and PHA-stimulated cells yielded fivefold and ninefold increases in tissue factor, respectively, as compared with control cells incubated without antigen or mitogen. Stimulation appeared as early as 4 h after the addition of either PHA or PPD and was maximal at 24 h incubation. Significantly increased levels of tissue factor were demonstrated in the stimulated cells (compared with control cells) at both 48 and 72 h, but the activities had returned to control levels by 96 h. Similar results were obtained in several cultures of lymphocytes from patients with hemophilia A. A representative 24 h harvest of cells yielded 42 U of tissue factor from the PHA-stimulated cells and only 6 U from the control cells.

Using the unactivated PTT with human plasma as a substrate, comparable results were obtained with a very similar time course of generation during cell culture as was seen with tissue factor (Fig. 4). The apparent increased activity of the cells in this assay probably reflects species specificity in relation to the substrates

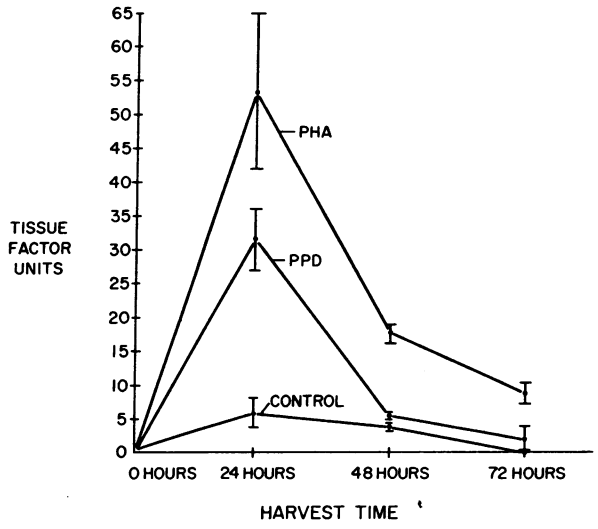

FIGURE 3 Time course of tissue factor activity from 80 lymphocyte cultures (control, $n = 35$; PHA, $n = 20$; PPD, $n = 25$). The horizontal axis indicates four time periods at which lymphocyte cultures were harvested as described in the Methods section. The vertical axis indicates tissue factor units as described by Nemerson (41). Control cells are unstimulated. Each point represents the mean (±1 SEM) of replicate determinations on each culture.

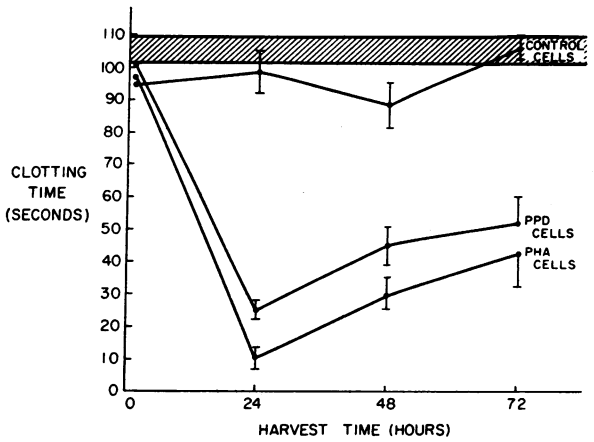

FIGURE 4 Time course of procoagulant activity from lymphocyte cultures. The horizontal axis again indicates the four time periods of harvest. The vertical axis indicates the clotting time of the samples in the unactivated PTT test, and the horizontal bar describes the range of the controls for this test (±1 SEM) using normal pooled plasma and buffer. Each point represents the mean (±1 SEM) of replicate determinations performed on the same cultures as described in Fig. 3.

TABLE II

Neutralization of Lymphocyte Coagulant Activity by Rabbit Antibody to Human Placental Tissue Factor

Sample	Sample + antibody serum* (1:1) (tissue factor units)		Sample + normal rabbit serum* (1:1) (tissue factor units)	
	Time 0	30 min	Time 0	30 min
Human brain (0.2 mg)	77.0	7.5	79.0	79.0
Control cells (24 h)	3.75	0	4.75	3.75
PHA cells (24 h)	27.0	0	23.5	19.0
PHA cells (48 h)	17.0	0	20.5	17.0
PHA cells (72 h)	10.5	0	9.5	8.5

* Preparation of the antibody and the normal rabbit serum control are described fully in Methods section.

since bovine plasma was used as the substrate in the tissue factor assay. Although it appeared that procoagulant activity persisted longer than tissue factor activity, the procoagulant activity was completely inhibited by incubation with the antibody to tissue factor.

In all experiments where tissue factor was generated by the lymphocytes, the material was completely inhibited by the rabbit antihuman tissue factor antibody (Table II). This antibody, previously characterized by Zeldis et al. (43), inhibited human brain tissue factor in either the standard tissue factor two-stage assay or the one-stage unactivated PTT. After 30 min of incubation with either the standard brain preparation or the lymphocytes, little or no residual activity was detected in the two-stage factor assay. Similarly, no procoagulant activity of the lymphocytes in the one-stage assay could be demonstrated following reaction with the antibody. In contrast to the aforementioned data regarding the lymphocyte procoagulant, granulocytes obtained from the bottom of the Ficoll-Hypaque gradient and handled in the same way as the lymphocytes demonstrated no tissue factor or procoagulant activity, regardless of harvest time. Trypan blue exclusion failed to show a significant difference in viability between stimulated and unstimulated cultures up to 5 days of incubation.

DISCUSSION

Though the liver has been shown to be the site of synthesis for most circulating coagulation factors (47, 48), the site of production of factor VIII is unknown. Perfusion studies of livers and spleens (23–25) have revealed variable amounts of factor VIII activity in the perfusates. More recent work has shown that the perfusate of rabbit livers contains a material that can stimulate a perfused spleen to release increased levels of factor VIII activity (26), thus supporting the postulate that the spleen has an active role in the synthesis of factor VIII. Dodds, Raymond, Moynihan, and Fenton have additionally suggested that the hepatic production of factor VIII might be regulated by this same material as indicated by increasing factor VIII activity from the liver with prolonged perfusion (26). The roles of the liver and spleen in factor VIII synthesis have been further defined by organ transplantation in animals and humans. Variably increased levels of factor VIII activity have been demonstrated in hemophilic subjects who received either liver or spleen transplants (28–31). Similarly, infusion of spleen cell concentrates resulted in an increase of factor VIII levels of six patients with hemophilia A (27). Tissue homogenates from both normal dog kidneys and from a strain of hemophilic dogs have also been shown to have factor VIII-like activity (49). However, transplantation experiments (50) and perfusion studies (48) have failed to support the thesis that the kidney synthesizes significant quantities of factor VIII.

Cell culture studies have described factor VIII synthesis by leukocytes (1), human skin fibroblasts, monkey kidney cells and liver cells (51), and by splenic macrophages (52). Bouhasin, Monteleone, and Altay reported a case of a patient with hemophilia A who developed acute lymphoblastic leukemia and subsequently had normal factor VIII levels measured (2). In this study, lymphocytes from seven normal patients were reported to synthesize factor VIII in culture after exposure to PHA, but the leukemic cells were not cultured.

All of the previously described experiments have stimulated great interest in the concept of a multifocal site of origin of factor VIII and supported the older hypothesis that a reticuloendothelial cell was responsible (53) for its synthesis. More recent experiments by de Los Santos and Hoyer utilizing immunofluorescent techniques have shown localization of factor VIII antigen to the endothelial cells of most organs (54), but the question of storage versus synthesis remains unanswered. With the exception of the previously mentioned study (54), none of the earlier experiments have proven conclusively that the material assayed was specifically factor VIII. Therefore, it was not surprising that we were unable to demonstrate factor VIII antigen in the lymphocyte preparations in spite of the activity observed in the factor VIII coagulation assay. The evidence that the factor VIII antibody used in our experiments was specific for factor VIII has been summarized elsewhere (44, 45). These data support the conclusion that the antibody binds to antigen sites on the same molecule as that responsible for factor VIII procoagulant activity.

The data presented here confirm that human blood lymphocytes contain the procoagulant material tissue factor rather than factor VIII. Similar results from human fibroblasts have been presented by Green, Ryan, Malandruccolo, and Nadler (55). The failure of other investigators to find procoagulant activity in cultures of either

rabbit spleen lymphocytes (52) or canine lymphocytes (56, 57) may merely reflect either species differences or methodological variations. We found almost no procoagulant activity released into the supernatant media of the cultures and therefore used lysates of cells exclusively. Previous studies have relied on assays of the supernatants or have utilized incompletely disrupted cells. Further, we cultured the lymphocytes in the presence of either a potent mitogen (PHA) or an antigen (PPD) to which the cells would predictably respond by undergoing blastic transformation. Therefore, we are describing an event that parallels other known functions of stimulated lymphocytes. That is, cells were evaluated not in the dormant state described by other authors but in a dynamic situation perhaps analogous to that described for leukocytes challenged with endotoxin (13, 18).

That tissue factor is an ubiquitous coagulation protein seems well supported by the finding of stainable tissue factor in the plasma membranes of many types of cells (43). It appears that interaction of cell membranes with a variety of stimuli (58, 17) permits either the liberation of preformed material or the synthesis of new tissue factor. Lerner, Goldstein, and Cummings (17) and Niemetz (18, 59) have demonstrated that the addition of acinomycin D, puromycin, or cyclohexamide in vitro markedly inhibited the generation of leukocyte procoagulant activity in response to endotoxin. They have suggested that endotoxin stimulates the production of tissue factor in the cells and is dependent upon protein synthesis. In the absence of immunologic or biochemical measures of tissue factor protein it is difficult to evaluate this proposal. An alternative hypothesis suggests that membrane-bound tissue factor is activated by various stimuli so that either the complete protein is formed or, alternatively, an active procoagulant site is exposed by cleavage of a large molecule. Preliminary data suggest that cells without coagulant activity do indeed contain tissue factor antigen.

The pathophysiological importance of lymphocyte tissue factor has yet to be determined, but it is intriguing to consider the various disease entities characterized both by lymphocytic infiltration and pathological thromboses. It is apparent that an understanding of the interaction of various stimuli with cell membranes is crucial for an interpretation of these findings. Studies are presently in progress to define better the character of antigen-lymphocyte interactions with regard to tissue factor elaboration.

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