Inhibition of the Activation of Hageman Factor (Factor XII) by Peripheral Blood Cells

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Abstract

Suspensions of peripheral blood mononuclear cells (PBMC), monocytes, T or B lymphocytes, platelets or granulocytes, and cell-depleted supernatant fluids of these suspensions inhibited activation of Hageman factor (HF, Factor XII) by ellagic acid, a property not shared by erythrocytes. PBMC also inhibited HF activation by glass or sulfatides. Contaminating platelets may have contributed to inhibition by PBMC.

Elaboration of agents inhibiting HF activation required metabolically active cells. The inhibitor(s) in PBMC supernates were not identified with known agents, but had properties of a nonenzymatic protein. PBMC supernates did not contain fibrinogen, nor alter the thrombin, prothrombin, or partial thromboplastin times of normal plasma, amidolysis by activated plasma thromboplastin antecedent (Factor XIa) or activated Stuart factor (Factor Xa) or esterolysis by C$\bar{1}$ (C1 esterase); they inhibited plasmin minimally.

These experiments suggest that peripheral blood cells may impede intravascular coagulation. Whether this property helps maintain the fluidity of blood is unclear.

Introduction

A variety of devices appear to maintain the fluidity of circulating blood. Among these are a multitude of plasma proteins that inhibit the enzymatically active forms of clotting factors or, under suitable circumstances, digest procoagulant proteins or the fibrin clot itself. The experiments to be described suggest that another class of substances may deter the formation of intravascular clots. Suspensions of peripheral blood mononuclear cells, granulocytes, and platelets all appear to inhibit the activation of Hageman factor (HF,[1] Factor XII). The responsi-

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1. *Abbreviations used in this paper:* BS, barbital-saline buffer; BS-Alb, BS containing 0.25% bovine albumin; C$\bar{1}$, C1 esterase, activated form of the first component of complement; C$\bar{1}$-INH, C1 esterase inhibitor; C1q, the C1q subcomponent of the first component of complement; DFP, diisopropylfluorophosphate; DPBS, Dulbecco's phosphate-buffered saline; DPBS-CaMg, Dulbecco's phosphate-buffered saline without calcium and magnesium ions; HF, Hageman factor, Factor XII; HPPAN, H-D-prolyl-L-phenylalanyl-L-arginine *p*-nitroanilide hydrochloride (S2302); LBTI, lima bean trypsin inhibitor; PF4, platelet factor 4; *p*-NA, *p*-nitroaniline; PTA, plasma thromboplastin antecedent (Factor XI); SBTI, soybean trypsin inhibitor; S2222, benzoyl-isoleucyl-glutamyl-glycyl-arginine *p*-nitroanilide; S2238, H-D-phenylalanyl-pipecolyl-arginine-*p*-nitroanilide.

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ble agent or agents are detected in cell-depleted supernatant fluid separated from cell suspensions. The inhibitory properties are associated with proteins that are synthesized by the cells. The biological significance of the inhibitory agent or agents in deterring intravascular coagulation remains to be determined.

Methods

Heparinized blood was prepared by drawing 50 ml blood from the antecubital vein of human subjects into polypropylene syringes containing 1 ml heparin (1,000 NIH U/ml); the donors were normal adults except for a 19-yr-old woman with idiopathic aplastic anemia (5,000 platelets/μl). Citrated blood was prepared by transferring 10 ml normal venous blood, drawn into a polypropylene syringe, to a polystyrene centrifuge tube containing 0.2 ml sodium citrate buffer (0.5 M, pH 5.0). Human AB serum was separated from venous blood of fasted donors that was incubated overnight in glass at 37°C. Pooled normal plasma, arbitrarily said to contain 1.00 U HF/ml, was prepared from the venous blood of 24 normal men aged 21 to 40 yr and stored at −70°C (1). Informed consent was obtained from all subjects.

HF (Factor XII) was purified by two different methods (2, 3), and was devoid of other clotting factors and plasminogen. It displayed a single band upon unreduced or reduced polyacrylamide gel electrophoresis. Before use, HF was diluted, usually to 0.05 U/ml, in 0.25% bovine serum albumin in barbital-saline buffer (BS-Alb).

Apparent solutions of 10^{-4} M ellagic acid (synthesized by Dr. James Crum in 1966 or Dr. Miklos Bodanszky in 1983, Case Western Reserve University) in BS were prepared as described earlier (2) and diluted to the desired concentration with BS.

The buffers used included BS (2), BS-Alb, tris-imidazole-saline buffer (0.025 M, pH 8.2) (4), Dulbecco's phosphate-buffered saline (DPBS) or DPBS without calcium and magnesium ions (DPBS-CaMg) (MA Bioproducts, Walkersville, MD; KC Biological, Lenexa, KA; or Gibco, Grand Island, NY) (5), DPBS with 1% bovine albumin (DPBS-Alb), RPMI 1640 with 2 mM L-glutamine (RPMI) (6), RPMI with 10% human AB serum (RPMI-HS), and RPMI with 10% fetal bovine serum (RPMI-FBS) (MA Bioproducts).

Sheep erythrocytes, suspended in Alsever's solution, were sedimented at 1,800 g at 10°C, washed three times with RPMI and suspended at a concentration of 1% (vol/vol) in RPMI.

FBS (Sterile Systems, Logan, UT) was heated at 56°C for 30 min before use.

H-D-prolyl-L-phenylalanyl-L-arginine *p*-nitroanilide dihydrochloride (HPPAN, S2302), benzoyl-isoleucine-glutamyl-glycyl-arginine *p*-nitroanilide (S2222), H-D-valyl-L-leucyl-L-lysine *p*-nitroanilide (S2251), and H-D-phenylalanyl-pipecolyl-arginine *p*-nitroanilide (S2238) were obtained from Helena Laboratories (Beaumont, TX), dissolved at a concentration of 1 mM in distilled water, and stored at 4°C. Before use, HPPAN and S2238 were diluted with an equal volume of Tris-imidazole-saline buffer. *N*-Acetyl-L-tyrosine ethyl ester (Calbiochem, La Jolla, CA) was dissolved at a concentration of 1 M in ethyleneglycol monomethyl ester (Fisher Scientific, Fair Lawn, NJ).

Crude IgG fractions of normal goat and rabbit sera (Pel-Freez Biologicals, Rogers, AR), rabbit antiserum against human C1q (Boehringer-Mannheim Biochemicals, Houston, TX) and goat antiserum against β_2-glycoprotein-I (Calbiochem) were separated as de-

scribed earlier (7, 8). Crude IgG fractions of normal goat and rabbit sera, goat antiserum against human platelet factor 4 (PF4; Atlantic Antibodies, Scarborough, ME) and rabbit antiserum against human CĪ-INH (CĪ inactivator, Behring Diagnostics, La Jolla, CA) were prepared in the same way and further purified by filtration through DEAE-Affigel Blue (Bio-Rad Laboratories, Richmond, CA) and concentrated, first by negative pressure dialysis against BS and then in a Centricon 30 microconcentrator (Amicon Corp., Danvers, MA).

Other reagents used included heparin (beef lung, 100 U/ml, Upjohn Co., Kalamazoo, MI), Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ), crystallized bovine serum albumin (BSA, Pentex, Miles Laboratories, Naperville, IL), bovine brain sulfatides (monogalactosyl-stearate, Supelco, Bellefonte, PA), bovine thrombin (Thrombostat, U. S. Pharmacopeia, Parke-Davis Co., Detroit, MI), and bovine trypsin (Worthington Biochemical Corp., Freehold, NJ). EDTA, lima bean trypsin inhibitor (LBTI), soybean trypsin inhibitor (SBTI), diisopropylfluorophosphate (DFP), benzamidine hydrochloride, cycloheximide and sodium azide were from Sigma Chemical Co., St. Louis, MO. Purified human Stuart factor (Factor X), activated by Russell's viper venom (Factor Xa) (9) and purified human trypsin-activated plasma thromboplastin antecedent (activated PTA, Factor XIa) (10) were the gifts of Dr. L. T. Goodnough, Case Western Reserve University. PF4 was the gift of Dr. L. Culp, Case Western Reserve University (11). CĪ (C1 esterase) was prepared as described by Donaldson (12). Crude soybean phosphatides (Centrolex "P," the gift of Central Soya Co., Fort Wayne, IN) were suspended at 0.1% in 0.15 M sodium chloride.

Peripheral blood cells were separated by modifications of published methods, as described below. Before testing, the supernatant fluids of cell suspensions were filtered through Millex GV 0.22 µm filters (Millipore Corp., Bedford, MA), as described below.

Peripheral blood mononuclear cells (PBMC) and the supernatant fluids of PBMC suspensions were prepared by an adaptation of Böyum's method (13). Typically, in replicate, 25 ml heparinized blood was diluted with 25 ml DPBS-CaMg. 10-ml aliquots were layered onto 3 ml Ficoll-Paque, and centrifuged at 450 g for 45 min. The PBMC layers were combined, washed twice with 40 ml DPBS-CaMg, once with 40 ml of an equal mixture of DPBS-CaMg and DPBS and once with 40 ml of DPBS, pelleting the cells each time at 500 g for 10 min. The washed cells, suspended in DPBS at 5 × 10⁶/ml, were recentrifuged in the same way. The mean viability of PBMC was 93% (SD±6.8%).

The supernatant fluid was filtered through a Millex GV 0.22-µm filter unit attached to a polypropylene syringe, replacing the filter after 15 ml had been filtered. The PBMC, resuspended at 5 × 10⁶ cells/ml, and the filtered supernatant fluid were saved. No lactic dehydrogenase was detected in the PBMC supernate in a sample containing 15 µg protein/ml, using a method sensitive to 1 U/liter (14). In some experiments, the cells were recentrifuged and resuspended repeatedly, saving successive supernatant fluids. PBMC and their supernates were tested within an hour of separation after storage in an ice-water bath.

Concentrates of the supernatant fluid of PBMC were prepared by negative pressure dialysis against DPBS, or by positive pressure filtration through an Amicon filter (ultrafiltration cell, model 8400, Amicon Corp.) fitted with a PM 10 membrane. Concentrates were made from the combined supernates of replicate preparations or from the combined supernates of successive washes of a single preparation of PBMC.

The possible presence of CĪ-INH, C1q, β_2-glycoprotein-I or PF4 in concentrated PBMC supernatant fluid (1 mg protein/ml) was examined by immunodiffusion, using 1% agar (15). The presence of glycosaminoglycans containing uronic acid was tested by measuring the concentration of uronic acid in the same concentrate (16).

The suspensions of PBMC were contaminated with platelets. In some experiments, the proportion of platelets was reduced by ~ 85–95% by centrifuging 25 ml of heparinized blood at 200 g for 15 min. The platelet-rich supernate was removed, and the cells were washed three times with DPBS-CaMg, centrifuging at 200 g. The platelet-depleted blood was used to prepare PBMC.

To test the effect of trypsin on the inhibitory properties of PBMC and PBMC supernate, 0.025 ml bovine trypsin (0.05 mg/ml BS) or BS was incubated with 0.1 ml PBMC, supernate or DPBS at 37°C for 2 h in 12 × 75 mm polystyrene tubes. Thereafter, 0.025 ml LBTI (0.2 mg/ml BS) and 0.05 ml HF (0.1 U/ml in 0.5% BS-Alb) or BS-Alb alone, were added in rapid succession. After 10 min, the amidolytic activity of HF was measured as described below.

Ammonium sulfate-precipitable material in PBMC supernates was separated by addition of sufficient solid ammonium sulfate to provide 60% saturation. The mixture was incubated at 4°C overnight and the precipitate that formed, separated by centrifugation at 2°C for 15 min at 2,000 g, was dissolved in sufficient DPBS to restore the original volume and dialyzed for 1 h against running tap water and overnight against ~ 100 vol of DPBS.

DFP-treated PBMC supernatant fluids were prepared by adding 1/100th vol 0.1 M DFP in isopropanol (final concentration 1 mM). The mixture was incubated for 90 min and then dialyzed against 100 vol DPBS at 4°C overnight. As a control, the supernates were treated in the same way with 1/100th vol of isopropanol.

Cycloheximide-treated PBMC were prepared by suspending PBMC in RPMI at a concentration of 5 × 10⁶ cells/ml. To one of two 12-ml aliquots, 0.12 ml cycloheximide (5 mg/ml DPBS) was added to provide a concentration of 50 µg/ml (18 µM). The two aliquots were incubated with mild agitation for 16 h at 37°C and the cells tested for viability. The aliquots were centrifuged for 10 min at 500 g and washed three times in DPBS. The pelleted cells were diluted in DPBS to 5 × 10⁶/ml, recentrifuged, the supernatant fluid saved, and the cells resuspended in DPBS at 5 × 10⁶/ml.

Sodium azide-treated PBMC were prepared by suspending PBMC in RPMI at 10⁷ cells/ml. 9.0-ml aliquots were diluted with 9.0 ml sodium azide (10 mg/ml RPMI) or RPMI alone, incubated with mild agitation for 20 h at 37°C, washed as described for cycloheximide-treated cells, and tested for viability.

Crude cytosol and particulate fractions of PBMC were separated from 5 ml washed PBMC in DPBS (5 × 10⁶ cells/ml), snap-frozen in polypropylene vials in a dry ice-ethanol mixture and thawed at 37°C, repeating the process twice more. The mixture was centrifuged at 100,000 g for 1 h at 2°C, the supernatant cytosol fraction was separated, and the particulate fraction was resuspended in 5 ml DPBS and recentrifuged. The supernatant solution was withdrawn and the particulate fraction was suspended in 5 ml DPBS.

A monocyte-enriched fraction of PBMC was prepared by a modification of the method of Fischer and Koran (17). PBMC were separated from platelet-depleted normal blood, substituting 10 ml RPMI containing 2 mM L-glutamine and 10% human AB serum for DPBS in the final step. The cells were diluted to 5 × 10⁶/ml in the same buffer. Quadruplicate aliquots of 11 ml were plated onto 75 cm² polystyrene tissue culture flasks (Corning Glassware, Corning, NY) and incubated for 70 min at 37°C in a humidified atmosphere of 5% carbon dioxide and 95% air. The flasks were then washed three times with 5.0 ml DPBS to remove nonadherent cells. Adherent cells were harvested by adding 5.0 ml DPBS and scraping the flasks with a rubber policeman, transferring the cell suspension to a 50-ml polypropylene tube. The combined cells suspensions of the replicate tubes were centrifuged at 600 g for 15 min. The sedimented cells, suspended at 5 × 10⁶/ml in DPBS, were recentrifuged, the supernatant fluid was removed and saved, and the cells were resuspended at the same concentration. Crude cytosol and particulate fractions of monocytes were prepared as described for PBMC.

A human monocytoid cell line (U937), derived from a human histiocytic lymphoma, was cultured by the method of Sundstrom and Nilsson (18). The cells were washed and centrifuged for 10 min at 500 g in polystyrene tubes, the cells were then suspended successively, twice in DPBS-CaMg, once in an equal mixture of DPBS-CaMg and DPBS, and finally in DPBS, separating the cells each time by centrifugation. The final cell suspension, > 95% viable, was diluted in DPBS to 5 × 10⁶/ml, and recentrifuged and the supernatant fluid saved.

Lymphocytes were prepared in the same manner as the monocyte-

enriched cell fraction by centrifuging nonadherent PBMC at 500 g for 10 min. The cells were washed three times in DPBS, diluted to 5 × 10⁶/ml with DPBS and resedimented at 500 g for 10 min. The supernatant fluid and the sedimented cells, resuspended in DPBS to the same concentration, were saved. Crude cytosol and particulate fractions of lymphocytes were separated as described for PBMC.

T and B lymphocytes were prepared by modifications of the methods of Greaves and Brown (19) and Weiner et al. (20). In brief, a cell fraction enriched in T cells was separated from a preparation of PBMC, substituting RPMI-HS for the final addition of DPBS. The cells were diluted to 10⁷/ml in RPMI-HS. 6 ml were transferred to each of replicate polystyrene tissue culture flasks and incubated at 37°C for 1 h in a humidified atmosphere of 5% carbon dioxide and 95% air. The suspensions of nonadherent cells were removed, each flask was rinsed twice with 2.5 ml prewarmed RPMI-FBS, and the combined suspensions were centrifuged at 500 g for 10 min. The sedimented cells, suspended in 6.5 ml RPMI-FBS, were filtered through 600 mg acid-washed nylon wool (scrubbed nylon, Fenwal Laboratories, Deerfield, IL) wetted with RPMI-FBS, refiltering the cells through the same column three times. Nonadherent cells were filtered through the column by applying 10 ml RPMI-FBS, prewarmed to 37°C. The effluents of three such columns were combined, centrifuged at 500 g for 10 min, the cell pellet was suspended in 6.5 ml DPBS, recentrifuged, and the pellet washed three more times with 20 ml DPBS. The cell suspension, diluted to 5 × 10⁶ cells/ml, was recentrifuged, the supernatant fluid was saved and the cells were resuspended to 5 × 10⁶/ml. The proportion of T cells was identified immunologically by cytofluorography, using mouse monoclonal antibodies to the OKT 11 marker (Ortho Diagnostic Systems, Raritan, NJ) (21).

A fraction of PBMC enriched in B cells was separated by washing the nylon wool column used to separate T cells with 50 ml RPMI-FBS and allowing the column to drain. Adherent B cells were dislodged mechanically, eluted with 5 ml RPMI, and centrifuged at 500 g for 10 min at 10°C. The pelleted cells were suspended in 1 ml of RPMI-FBS, and diluted to 3 × 10⁶ cells/ml. 1 ml of this crude B cell suspension was mixed with 1 ml of a 1% suspension of sheep erythrocytes in RPMI, centrifuged at 500 g for 5 min at 4°C, and incubated at 4°C overnight. The pelleted cells were resuspended and the contents of five such tubes were layered over Ficoll-Paque at a ratio of 8 ml cell suspension and 3 ml Ficoll-Paque. The tubes were centrifuged at 500 g for 30 min, the separated cell layer, enriched with B cells, was washed three times in DPBS, and the cells were diluted to 5 × 10⁶/ml and recentrifuged. The supernatant fluid was saved and the cells were resuspended in DPBS. The proportion of B cells was identified by cytofluorography, using goat polyvalent immunoglobulin against surface immunoglobulin (PV antibodies, Kallestad Laboratories, Minneapolis, MN) (21).

Granulocytes were separated from 25 ml heparinized venous blood by a modification of published methods (13, 22). The blood was diluted with an equal volume of DPBS-CaMg, layered over 18 ml Ficoll-Paque, and centrifuged at 450 g for 40 min. The supernatant fluid was removed and the thin leukocyte layer, ~ 3 ml, was transferred to each of two 50-ml sterile polystyrene centrifuge tubes. A volume of 24 ml of distilled water was added to each tube and, after 20 s, 8 ml of 0.67 M (3.6%) sodium chloride was added rapidly to each tube and the contents mixed. The tubes were centrifuged at 250 g for 10 min at 2°C, the pelleted granulocytes were resuspended in 24 ml water and the process repeated. The cells were then suspended in 10 ml DPBS-CaMg and rewashed, twice with DPBS-CaMg, once with an equal mixture of DPBS and DPBS-CaMg, and finally with DPBS. The pelleted cells were suspended in DPBS at 5 × 10⁶/ml. The cell suspension and the supernatant fluid after centrifugation were saved.

Erythrocytes were separated from 10 ml citrated venous blood by centrifugation at 250 g for 15 min. The supernatant, platelet-rich plasma was withdrawn and 4 ml of the pelleted erythrocytes were aspirated from the bottom of the tube, recentrifuged, and the bottom 3 ml of erythrocytes were withdrawn and washed three times with 3 ml DPBS, sedimenting the cells each time at 2,000 g for 10 min. The erythrocytes were then diluted to 2 × 10⁷ cells/ml with DPBS and was

recentrifuged at 2,000 g for 10 min. The supernatant fluid was removed and saved, and the erythrocytes were rediluted to 2 × 10⁷ cells/ml. No platelets or leukocytes were seen in smeared preparations stained with Wright-Giemsa stain.

Platelets were separated from 40 ml citrated blood by a modification of Kinlough-Rathbone's (23) method. The platelet-rich plasma, sedimented at 200 g for 10 min, was recentrifuged at the same speed to reduce contamination with erythrocytes. The platelet-rich plasma was mixed with an equal volume of DPBS-Alb, incubated at 37°C for 15 min and centrifuged at 1,800 g for 10 min. The pelleted platelets were washed twice more in 10 ml of DPBS-Alb and suspended in DPBS (without albumin) at 10⁸ cells/ml. This suspension and the supernatant fluid after recentrifugation were saved. No erythrocytes or leukocytes were seen in smears of the platelet suspension stained with Wright-Giemsa stain.

The effect of cell suspensions or the supernatant fluid of cells suspensions on activation of HF by ellagic acid was measured by incubating 0.1 ml HF (0.05 U/ml in BS-Alb) with 0.1 ml of the agent to be tested, diluted serially with DPBS, for 10 min at 37°C in 12 × 75 mm polystyrene tubes. Thereafter, 0.05 ml ellagic acid (2 × 10⁻⁵ M in BS) was added to each tube. Incubation was continued for 60 min, after which 1.0 ml HPPAN was added. Incubation was continued for 60 min and the reaction was then stopped by addition of 0.3 ml glacial acetic acid. The amount of p-nitroaniline (p-NA) released from HPPAN was measured spectrophotometrically at 405 nm against blanks in which glacial acetic acid was added before substrate, in comparison to the OD of p-NA solutions. Mixtures containing cells were centrifuged at 800 g for 10 min before measurement of OD. Controls included the substitution of appropriate buffers for the test solution, HF, or ellagic acid. The degree of inhibition by the test solutions was determined in comparison to the amidolytic activity of a range of concentrations of HF. The coefficient of variation of the amidolytic assay of HF was 3.2% (SD±3.1%, SE±1.0%) of any given value excluding observations in which the OD was 0.010 or less. The coefficient of variation of the assay for inhibitory activity was 3.7% (SD±3.4%, SE±0.6%). Variations of this technique are described in the text. All experiments were performed in replicate.

To test the effect of LBTI and SBTI on PBMC supernates, 0.1 ml of supernate was incubated with 0.05 ml LBTI or SBTI in BS, serially diluted with BS, for 10 min at 37°C, 0.05 ml HF (0.1 U/ml BS-Alb) was added and incubation was continued for 10 min, after which 0.05 ml 2 × 10⁻⁵ M ellagic acid was added. After an additional 60 min, 1.0 ml HPPAN and 0.05 ml BS were added, and the assay continued as described above. As controls, BS was substituted for LBTI or SBTI in the initial mixture, and LBTI or SBTI was substituted for BS in the final mixture.

The effect of the supernatant fluid of cell suspensions on the activation of HF by glass was measured by mixing 0.25 ml HF (0.05 U/ml BS-Alb) or bovine albumin alone and 0.25 ml supernatant fluid, diluted serially with DPBS, or DPBS alone. Aliquots of 0.2 ml were transferred to 10 × 75 mm disposable borosilicate glass tubes (Fisher Scientific Co., Pittsburgh, PA). The tubes were incubated for 60 min at 37°C, agitating the tubes continuously in a mechanical shaker. Thereafter 0.1 ml DPBS and 1.0 ml HPPAN were added and incubation and shaking were continued for 60 min, after which 0.3 ml glacial acetic acid was added. The amount of p-NA released was measured as described above.

The capacity of PBMC supernates to inhibit the clot-promoting properties of glass was tested by an earlier method (24). In duplicate, 10 × 75 mm disposable glass tubes were coated with 0.2 ml samples of supernate or BS. Volumes of 0.1 ml each of normal pooled plasma and Centrolex P were incubated in these tubes at 37°C for 1 min, and the clotting time was measured at this temperature after addition of 0.1 ml 0.025 M calcium chloride, prewarmed at 37°C.

To determine whether binding took place between HF and the inhibitory agent(s) in PBMC supernates, a concentrate of PBMC supernate was bound covalently to cyanogen bromide-activated agarose (CNBr-activated Sepharose 4B, Pharmacia Fine Chemicals) according

to the manufacturer's directions, at a ratio of 10 mg protein to 1 g gel. A control gel was prepared in the same way, omitting addition of PBMC supernate and blocking the active site with ethanolamine. A volume of 2 ml of gel was packed in polypropylene Econo-columns (i.d. 0.8 cm; Bio-Rad Laboratories) and washed with DPBS before use. 1 ml HF (1.0 U/ml BS-Alb, 48 U/mg protein) was filtered through each column, eluting with DPBS. 1-ml aliquots were collected in polystyrene tubes and assayed for coagulant HF activity in comparison with that of pooled normal plasma (25).

The effect of PBMC supernates on the coagulant action of thrombin was tested in 10 × 75 mm polystyrene tubes by measuring the clotting time at 37°C of a mixture of 0.1 ml each of pooled normal human plasma, a concentrate of supernate in DPBS, and bovine thrombin (5 U/ml). The presence of fibrinogen in supernates was examined in the same way, omitting plasma. The effect of PBMC supernates on the prothrombin time and kaolin-activated partial thromboplastin time was similarly measured (25).

The effect of PBMC supernates on the amidolytic properties of activated PTA (26) and plasmin (25) and the esterolytic properties of C$\bar{1}$ (27) was determined by published methods. The effect of PBMC supernates on the amidolytic properties of activated Stuart factor was measured by an adaptation of method I of Ødegard et al. (28), incubating 0.2 ml concentrated PBMC supernate (1.5 mg protein/ml DPBS) or DPBS alone at 37°C for 5 min with 0.1 ml activated Stuart factor (2 U/ml 0.25% bovine albumin in BS, specific activity 1,200 U/ml protein), in 12 × 75 mm polystyrene tubes. Thereafter, 0.2 ml 10^{-3} M S2222 was added, incubation was continued for 7 min, and the reaction was stopped by addition of 0.3 ml glacial acetic acid. The amount of p-NA released was measured at 405 nm in comparison to blanks to which acetic acid was added before substrate.

Cell counting was carried out with a Coulter counter (model ZM, Coulter Electronics, Hialeah, FL). The proportion of monocytes and lymphocytes in suspensions of PBMC was estimated by histochemical staining of monocytes for nonspecific esterase (17). Differential counts of granulocytes were performed by Wright-Giemsa staining of smeared cells. T cells and B cells were identified with an automated flow-cytometry system (Coulter Epics V, Coulter Electronics) (21). Cell viability was tested by trypan blue exclusion (29).

Protein assays were performed by the method of Lowry (30).

All centrifugations were at room temperature unless otherwise stated, at low speeds in a refrigerated centrifuge (model PR-2, Damon/International Centrifuge Co.,) at 2°C and at room temperature in a centrifuge (model UV, Damon/International Centrifuge Co.). Ultracentrifugation was performed in a centrifuge (model L5-65; Beckman Instruments, Inc., Fullerton, CA) at 2°C.

Dialyses were performed in Spectro-Por 2 cellulose casings (6.4 mm diam molecular weight cut-off 12–14,000; Spectrum Medical Industries, Los Angeles, CA).

Results

The inhibitory effect of PBMC suspensions and their supernatant fluids on the activation of HF by ellagic acid. Suspensions of PBMC inhibited the activation of HF by ellagic acid, as measured by amidolysis of HPPAN. In a typical experiment, PBMC, at initial concentrations of 6.25 × 10⁵ cells/ml or more, significantly inhibited activation of HF (Table I). Similarly, the supernatant fluid of PBMC cell suspensions inhibited activation of HF. Inhibitory properties for the activation of HF were detected in variable but essentially similar amounts in the supernatant fluids of nine further washes of the PBMC suspension (data not shown). The inhibitory activity of different supernatant fluid preparations was roughly proportional to their protein content; a dilution of supernate that inhibited half the amidolytic activity of the HF in a mixture of HF and supernate had a protein content averaging 7.3 μg/ml (SD±2.72 μg/ml).

The inhibitory properties of platelets, monocytes and lymphocytes on the activation of HF by ellagic acid. The PBMC used in the experiments described thus far were contaminated with platelets, containing, on the average, 13.3 (SD±7.0) platelets per PBMC (Table I). Platelet suspensions and their supernates inhibited activation of HF by ellagic acid, but this was significant only at relatively high concentrations of platelets (Table II).

Comparison of the data presented in Tables I and II suggests that a portion of the inhibitory effect of PBMC suspensions was due to their content of platelets. When PBMC were prepared from normal human blood that had been depleted of ~ 95% of its platelet content, inhibition of the activation of HF by PBMC was reduced by ~ 50%, and by the supernates of PBMC suspensions by ~ 75%. Thus, a significant portion of the inhibitory properties of PBMC suspensions could be attributed to platelets. That platelets were not solely responsible for the inhibitory properties of PBMC suspensions was demonstrated in studies of PBMC prepared from the blood of a patient with severe thrombocytopenia. At concentrations of platelets that were not inhibitory (Table II), both the cell suspension and the supernatant fluid inhibited activation of HF (Table I).

The PBMC were mixtures of ~ 80% lymphocytes and 20% monocytes. A monocyte-enriched fraction of PBMC, prepared from platelet-depleted blood, and the supernate of this fraction inhibited activation of HF (Table III). Similarly, the supernatant fluid of a suspension of cultured monocytoid cells (U937) inhibited activation of HF (Table III).

A lymphocyte-enriched fraction of PBMC, prepared from blood that had been depleted of platelets, and the supernate of this fraction inhibited the activation of HF (Table III). Both T and B lymphocytes and their supernates were inhibitory (Tables III and III).

The inhibitory properties of granulocytes and erythrocytes and their supernatant fluids on the activation of HF by ellagic acid. Granulocytes, separated as described, and the supernatant fluid of granulocyte suspensions inhibited activation of HF to approximately the same degree as PBMC suspensions (Table IV).

Neither erythrocytes nor the supernatant fluid of erythrocyte suspensions had significant inhibitory properties (Table IV).

Some properties of the inhibitory activity of PBMC supernates. The inhibitory properties of PBMC suspensions and their supernates were directed against the activation of HF and not at the amidolytic properties of activated HF. Addition of cells or supernatant fluid after HF had been incubated with ellagic acid for 60 min was without effect; the cells and supernatant fluid themselves did not have amidolytic properties for this substrate (Table I).

After ultracentrifugation, both the cytosol and particulate fractions of PBMC inhibited the activation of HF by ellagic acid, but in repeated experiments the cytosol appeared to be relatively more inhibitory (Table V). Similarly, both cytosol and particulate fractions of lymphocytes and monocytes were inhibitory (data not shown).

The inhibitory properties of PBMC supernates were not reduced by filtration of supernates through a Millex-GV filter, said to retain particles > 0.22 μm diam), nor by dialysis through casings with a mol wt cut-off of 12–14,000.

The inhibitory properties of PBMC supernates were re-

Table I. Effect of Peripheral Blood Mononuclear Cells on the Activation of HF by Ellagic Acid

Test mixture			Amidolysis*		
Cell suspension		Supernate dilution			
PBMC	Platelets		Cell suspension	Supernate	Buffer
cells/ml	*cells/ml*		*nmol p-NA released/ml/h*		
A. Normal blood					
5×10^6	9×10^7	Undilute	2.8	0.5	—
2.5×10^6	4.5×10^7	1/2	3.4	7.5	—
1.25×10^6	2.25×10^7	1/4	6.9	18.2	—
6.25×10^5	1.25×10^7	1/8	16.8	24.5	—
3.13×10^5	6.25×10^6	1/16	24.1	25.4	—
0.0	0.0	∞	—	—	27.6
$5 \times 10^{6\ddagger}$	$9 \times 10^{7\ddagger}$	Undilute‡	1.9	0.8	—
$5 \times 10^{6\S}$	$9 \times 10^{7\S}$	Undilute§	28.5	25.4	—
B. Blood of a patient with aplastic anemia					
5×10^6	2.7×10^6	Undilute	3.4	0.7	—
2.5×10^6	1.35×10^6	1/2	3.7	1.6	—
1.25×10^6	6.75×10^5	1/4	12.3	12.0	—
6.25×10^5	3.38×10^5	1/8	15.9	18.7	—
3.13×10^5	1.69×10^5	1/16	17.6	19.4	—
0.0	0.0	∞	—	—	21.4

* Amidolytic activity generated in a mixture of HF, test mixture (serially diluted with DPBS) and ellagic acid (see Methods). The test mixtures were PBMC (5×10^6 cells/ml DPBS), the supernatant of the PBMC suspension and DPBS alone (buffer). (*A*) The PBMC contained 9% monocytes and 91% lymphocytes, the platelet count in the undiluted PBMC suspension was 9×10^7 cells/ml or 18 platelets per PBMC, and the protein content of the supernate was 38 μg/ml. (*B*) PBMC were prepared from the blood of a patient with aplastic anemia (peripheral blood platelet count 5000/μl). The PBMC suspension contained 6% monocytes and 94% lymphocytes and the platelet count in the undiluted PBMC suspension was 2.7×10^6 cells/ml or approximately one platelet for each 2 PBMC. The protein content of the undiluted supernate was 16 μg/ml. In 12×75-mm polystyrene tubes, 0.1 ml HF (0.05 U/ml, 58 U/mg protein) and 0.1-ml test mixture were incubated at 37°C for 10 min, after which 0.05 ml 4×10^{-5} M ellagic acid was added and incubation was continued for 60 min. Thereafter, 0.1 ml DPBS and 1.0 ml HPPAN were added, incubation was continued for an additional 60 min, and the reaction was stopped by addition of 0.3 ml glacial acetic acid. The amount of p-nitroaniline (p-NA) that had been released was measured against suitable blanks to which glacial acetic acid had been added before HPPAN. ‡ Bovine albumin in BS substituted for HF in the original mixture. § DPBS substituted for PBMC or PBMC supernate in the original mixture and 0.1 ml PBMC or PBMC supernate added to the mixture instead of DPBS after the 60-min incubation of the mixture of HF and ellagic acid.

Table II. Effect of Platelets on the Activation of HF by Ellagic Acid

Test mixture		Amidolysis*		
Platelets	Supernate dilution	Platelets	Supernate	Buffer
cells/ml		*nmol p-NA released/ml/h*		
10^8	Undilute	12.4	3.4	—
5×10^7	1/2	19.0	9.8	—
2.5×10^7	1/4	20.9	18.0	—
1.25×10^7	1/8	23.6	22.0	—
6.25×10^6	1/16	24.6	25.2	—
0.0	∞	—	—	24.6

* Amidolytic activity generated in a mixture of HF, test mixture, and ellagic acid (see Methods). The test mixtures were suspensions of platelets (10^8 cells/ml), serially diluted with DPBS, the supernatant fluid separated from each suspension of platelets and DPBS alone. The assays were performed as described in the footnote to Table I. The specific activity of the HF was 58 U/mg protein.

tained after incubation at room temperature or 37°C overnight but were reduced by incubation at higher temperatures for 30 min by approximately one-fourth at 56°C and one-half at 60°C, and essentially completely by incubation in a boiling water bath for this time.

Binding of HF to the inhibitory agent(s) in PBMC supernates could not be demonstrated. The coagulant titer of HF was the same, whether filtered through a column of agarose to which PBMC supernate had been covalently linked, or through a control column of agarose.

Incubation of PBMC or PBMC supernates with trypsin reduced their inhibitory properties, suggesting that a protein component participated in inhibition of the activation of HF (Table VI). In agreement with this interpretation, approximately half of the inhibitory properties of PBMC supernates were precipitated by ammonium sulfate at 60% saturation.

No evidence was obtained that the inhibitory properties of PBMC supernatant fluids were enzymatic. The inhibitory properties of PBMC supernate appeared to be essentially instantaneous (Fig. 1). When PBMC supernate was incubated

Table III. Effect of Monocytes and Lymphocytes on the Activation of HF by Ellagic Acid

Test mixture		Amidolysis*		
Cells	Supernate dilution	Cells	Supernate	Buffer
cells/ml		nmol p-NA released/ml/h		
A. Adherent cells				
5×10^6	Undilute	0.6	0.3	—
2.5×10^6	1/2	0.1	0.0	—
1.25×10^6	1/4	0.6	6.4	—
6.25×10^5	1/8	13.5	21.8	—
0.0	∞	—	—	32.1
B. Monocytoid cells				
—	Undilute	—	1.5	—
—	1/2	—	3.8	—
—	1/4	—	11.5	—
—	1/8	—	20.9	—
—	∞	—	—	22.9
C. Nonadherent cells				
5×10^6	Undilute	0.0	0.0	—
2.5×10^6	1/2	1.0	2.6	—
1.25×10^6	1/4	10.3	19.6	—
6.25×10^5	1/8	19.3	25.6	—
0.0	∞	—	—	28.9
D. B cells				
5×10^6	Undilute	0.9	0.2	—
2.5×10^6	1/2	0.8	3.9	—
1.25×10^6	1/4	10.6	13.5	—
6.25×10^5	1/8	7.4	19.7	—
0.0	∞	—	—	19.8
E. T cells				
5×10^6	Undilute	0.0	1.3	—
2.5×10^6	1/2	0.7	11.8	—
1.25×10^6	1/4	1.6	18.2	—
6.25×10^5	1/8	8.0	21.3	—
0.0	∞	—	—	22.0

* Amidolytic activity generated in a mixture of HF, test mixture (serially diluted with DPBS) and ellagic acid (see Methods). The test mixtures in A were polystyrene-adherent cells, at an initial concentration of 5×10^6 cells/ml, prepared from PBMC that had been separated from platelet-depleted blood, and the supernate of the initial adherent cell suspension; the adherent cell suspension contained 94% monocytes, 6% lymphocytes and 2.3×10^7 platelets/ml or 4.6 platelets per adherent cell. The test mixture in B was the supernate of a suspension of human monocytoid cells (U-937) containing 5×10^6 cells/ml. The text mixtures in C were nonadherent cells, at an initial concentration of 5×10^6 cells/ml, prepared from PBMC that had been separated from platelet-depleted blood, and the supernate of the initial nonadherent cell suspension; the nonadherent cell suspension contained 1.5 percent monocytes, 98.5 percent lymphocytes, and 2.4 $\times 10^6$ platelets/ml, or one platelet per 2 nonadherent cells. The test mixtures in D and E were B and T cells and their supernates, respectively. The B cell preparation contained 85 per cent B cells, and the T cell preparation, 81 percent T cells. The assay was performed as described in the footnote to Table I. The specific activity of the HF used in A, C, D, and E was 58 U/mg protein, and in B, 98 U/mg protein. A, B, C, D, and E represent separate experiments.

Table IV. Effect of Granulocytes and Erythrocytes on the Activation of HF by Ellagic Acid

Test mixture		Amidolysis*		
Cells	Supernate dilution	Cells	Supernate	Buffer
cells/ml		nmol p-NA released/ml/h		
A. Granulocytes				
5×10^6	Undilute	0	3.3	—
2.5×10^6	1/2	0	8.9	—
1.25×10^6	1/4	3.5	15.9	—
6.25×10^5	1/8	4.2	18.2	—
3.12×10^5	1/16	11.8	20.1	—
1.56×10^5	1/32	15.8	20.3	—
0.0	∞	—	—	20.7
B. Erythrocytes				
2×10^7	Undilute	19.9	20.7	—
0.0	∞	—	—	21.5

* Amidolytic activity generated in 12 × 75 mm polystyrene tubes in a mixture of 0.1 ml HF (0.05 U/ml, 58 U/mg protein), 0.1 ml test mixture, serially diluted with DPBS, and 0.05 ml 2×10^{-5} M ellagic acid (see Methods). The test mixtures included neutrophils (5×10^6 cells/ml DPBS), erythrocytes (2×10^7 cells/ml DPBS), and the supernates of these cell suspensions. The granulocyte preparation contained 94% segmented neutrophils, 4% eosinophils and 2% lymphocytes. No platelets were discerned in stained smears of the granulocyte or erythrocyte suspensions. The protein content of the undiluted (100%) supernatant of the granulocyte suspension was 17μg/ml, and of the erythrocyte suspension, < 8 μg/ml.

with HF for varying intervals before addition of ellagic acid, its inhibitory activity gradually decreased. In contrast, when supernate was incubated with ellagic acid for a variable period

Table V. Effect of Cytosol and Particulate Fractions of PBMC on the Activation of HF by Ellagic Acid

Test mixture dilution	Amidolysis*			
	Cells	Cytosol	Particulate fraction	Buffer
	nmol p-NA released/ml/h			
Undilute	3.7	1.8	4.8	—
1/2	2.2	0.6	4.6	—
1/4	1.9	1.9	21.0	—
1/8	5.9	1.3	21.9	—
1/16	15.6	13.5	20.2	—
1/32	18.0	17.7	19.8	—
∞	—	—	—	20.4

* Amidolytic activity generated in 12 × 75 mm polystyrene tubes in a mixture of HF, test mixture (serially diluted with DPBS). The test mixtures were PBMC (5×10^6 cells/ml DPBS), the cytosol fraction of PBMC separated by ultracentrifugation of freeze-thawed PBMC, the particulate fraction after ultracentrifugation, diluted with PBMC to the original volume, and DPBS alone (buffer). The PBMC contained 20% monocytes and 80% lymphocytes. Amidolytic activity was measured as noted in the footnote to Table I. The specific activity of the HF was 100 U/mg protein.

Table VI. Effect of Trypsin on the Inhibitory Properties of PBMC and PBMC Supernate

Test mixture	Trypsin	HF	Amidolysis*
			nmol p-NA released/ml/h
DPBS	0	+	15.8
DPBS	+	+	17.8
DPBS	+	0	0
PBMC	0	+	4.6
PBMC	+	+	11.0
Supernate	0	+	5.1
Supernate	+	+	14.1

* In 12 × 75 mm polystyrene tubes, 0.1 ml DPBS or PBMC or PBMC supernate was incubated with 0.025 ml bovine trypsin (0.05 mg/ml) or BS at 37°C for 2 h. Thereafter, 0.025 ml LBTI, 0.2 mg/ml and 0.05 ml HF (0.1 U/ml) or BS-Alb alone were added in rapid succession and, after 10 min, 0.05 ml ellagic acid, followed, after 1 h, by 1 ml HPPAN. After an additional 60 min, the reaction was stopped by addition of 0.3 ml glacial acetic acid and the amount of p-nitroaniline (p-NA) released was measured against blanks to which glacial acetic acid was added before addition of substrate. The PBMC were suspended at a concentration of 5 × 10^6/ml DPBS; the supernate of PBMC was in the same buffer. Trypsin, LBTI, and ellagic acid were dissolved in BS. The specific activity of the HF was 100 U/mg protein.

before addition of HF, it retained its inhibitory properties for at least 60 min. At the concentrations tested, the inhibitory properties of PBMC supernatant fluids were not impaired by incubation with 1 mM DFP. Nor was inhibition of the activation of HF prevented by addition of 0.5 mM EDTA or 0.5 mM benzamidine to the initial mixture of HF and supernate. Appropriate concentrations of SBTI or LBTI are known to inhibit the amidolytic properties of ellagic acid-activated HF (31). At concentrations of 30 μg/ml or less in the mixture of PBMC supernate, HF and ellagic acid, concentrations of SBTI or LBTI that did not affect amidolysis by activated HF, these agents did not block the inhibitory properties of PBMC supernates.

The inhibitory properties of PBMC supernates could not be identified with other agents that might influence the activa-
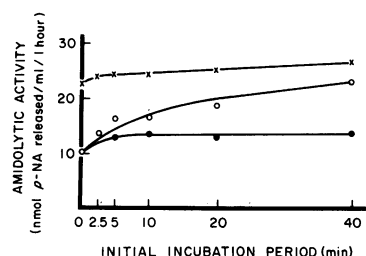
Figure 1. Time course of inhibition by PBMC supernates of the activation of HF by ellagic acid. In 12 × 75 mm polystyrene tubes, 0.1 ml PBMC supernate (diluted 50% with DPBS) was incubated for 10 s to 40 min with 0.05 ml ellagic acid (2 × 10^{-5} M), after which 0.1 ml HF (0.05 U/ml, 58 U/mg protein) was added. The mixture was incubated for an additional 60 min, 1.0 ml HPPAN was added, and incubation was continued for 60 min (●). Conversely, 0.1 ml supernate was incubated with 0.1 ml HF for 10 s to 40 min, after which 0.5 ml ellagic acid was added and the procedure continued as before (○). The reaction was stopped by addition of 0.3 ml glacial acetic acid and the amount of p-NA released was measured (see Methods). The amidolytic activity generated in mixtures of 0.1 ml HF and 0.1 ml DPBS, incubated for 10 s to 40 min before addition of ellagic acid, was also measured (×).

tion of HF. Thus, the thrombin time was unaltered in a mixture of 0.2 ml each of normal plasma, concentrated PBMC supernate (56 μg protein/ml) and bovine thrombin (5 U/ml), suggesting that the inhibitory effects of PBMC supernates were not attributable to heparinlike agents.

Supporting this interpretation, no uronic acid was detected in a concentrate of PBMC supernate containing 1 mg protein/ml. Moreover, the addition of PF4, at concentrations as high as 75 μg/ml in a mixture of PBMC supernate and HF did not neutralize the inhibitory properties of the supernate. Rather, as demonstrated in experiments in which its concentration was reduced to 0.24 μg/ml, PF4 itself inhibited the activation of HF by ellagic acid, and its inhibitory activity was additive to that of PBMC supernates.

The inhibitory properties of PBMC supernates could not be attributed to PF4, which could not be demonstrated upon immunodiffusion against antiserum against PF4; a crude IgG fraction of this antiserum did not neutralize the inhibitory properties of PBMC supernates (Table VII).

PBMC supernates did not contain material coagulable with thrombin. They did not alter the prothrombin time or activated partial thromboplastin time of normal plasma (data not shown). Nor did they inhibit the amidolytic properties of CĪ (data not shown); CĪ inhibitor was not demonstrable by immunodiffusion, and a crude immunoglobulin fraction of specific antiserum did not reduce the inhibitory properties of PBMC supernates (Table VII).

Table VII. Effect of Antiserums on the Inhibitory Properties of PBMC Supernate

	Amidolysis*
	nmol p-NA released/ml/h
A. Buffer	22.6
PBMC supernate + buffer	11.9
PBMC supernate + normal goat IgG	9.9
PBMC supernate + goat anti-Clq IgG	9.2
PBMC supernate + normal rabbit IgG	9.2
PBMC supernate + rabbit anti-β_2-glycoprotein-I IgG	10.6
B. Buffer	21.5
PBMC supernate + buffer	11.9
PBMC supernate + normal goat IgG	13.5
PBMC supernate + goat anti-PF4 IgG	14.7
PBMC supernate + normal rabbit IgG	12.9
PBMC supernate + rabbit anti-CĪ-INH	14.1

* In 12 × 75 mm polystyrene tubes, 0.05 ml PBMC supernate or DPBS (buffer) was incubated for 30 min at 37°C with 0.05 ml IgG or buffer, after which 0.1 ml HF (0.05 U/ml) was added, followed, after 10 min, by 0.05 ml ellagic acid. The evolution of amidolytic activity was then determined as described in Methods. (A) The specific activity of HF was 58 U/mg protein, the protein content of the PBMC supernate was 22 μg/ml and the initial concentration of ellagic acid was 2 × 10^{-4} M. (B) The specific activity of HF was 43 U/mg protein, the protein content of the PBMC supernate was 15 μg/ml, and the initial concentration of ellagic acid (of another lot) was 4 × 10^{-4} M. The initial protein concentration of IgG in A was 2.2 mg/ml, and in B, 1.2 mg/ml. As described in Methods, the IgG fractions used in A and B were separated by different techniques.

C1q and β_2-glycoprotein-I inhibit the activation of HF (32, 33). Neither of these proteins could be demonstrated in a concentrate of PBMC upon immunodiffusion against antisera against this protein. Further, a crude immunoglobulin fraction of these antiserums did not neutralize the inhibitory effect of PBMC supernates against the activation of HF (Table VII). At very high concentrations, PBMC supernates had some inhibitory activity against human urokinase-activated plasmin; a preparation of PBMC supernate (508 μg protein/ml) reduced the amidolytic activity of this enzyme from 37.0 to 29.4 nmol p-NA released/ml per h by the method described.

Incubation of PBMC for 16 h with cycloheximide (50 μg/ml) sharply reduced the inhibitory properties of PBMC supernates, which contained strikingly less protein than the supernates of untreated cells, as though protein synthesis had been depressed (Table VIII). Similarly, PBMC treated with cycloheximide were less inhibitory than untreated cells, but the difference was less pronounced.

Incubation of PBMC overnight with sodium azide at a

final concentration of 0.5 mg/ml, sufficient to kill 97% of cells, greatly reduced the inhibitory properties of both the cells and the supernatant fluid, which now contained no detectable protein (Table VIII).

The inhibitory properties of PBMC or PBMC supernates for the activation of HF by glass and by sulfatides. Conceivably, in the experiments recorded thus far, inhibition of the activation of HF may have been due to a specific interaction between PBMC or PBMC supernatant fluid and ellagic acid. The same inhibitory characteristics were, however, demonstrable when activation of HF was carried out by exposure to glass (Table IX). Consistently, at higher dilutions, PBMC supernates enhanced amidolysis, a property not observed in the absence of HF. This enhancement of amidolysis was not blocked by treatment of the supernates with 1.0 mM DFP (data not illustrated).

The plasmas of individuals with Hageman trait or plasma prekallikrein deficiency (Fletcher trait), and several ill-defined fractions or normal plasma, contain agents that adhere to glass and inhibit its capacity to initiate clotting (24, 34, 35). PBMC supernates, however, did not appear to share this property. Coating glass tubes with the plasma of a patient with Hageman trait lengthened the partial thromboplastin time of normal plasma from 79.5 s, tested in tubes coated with BS, to 107.8 s. In contrast, the clotting time of tubes coated with PBMC supernatant fluid (15 μg protein/ml) was 74.8 s.

Table VIII. Effect of Cycloheximide and Sodium Azide on the Inhibitory Properties of PBMC and Their Supernates

| Test mixture dilution | Amidolysis* | | | | |
	PBMC	Treated PBMC	Supernate	Treated supernate	Buffer
	nmol p-NA released/ml/h				
A. Cycloheximide					
Undilute	2.9	1.6	0.3	14.3	—
1/2	1.9	3.7	0.6	22.8	—
1/4	1.4	15.8	2.1	25.6	—
1/8	7.0	22.2	12.4	26.4	—
1/16	18.4	24.4	22.5	24.4	—
∞	—	—	—	—	27.4
B. Sodium azide					
Undilute	2.1	1.1	0.3	9.9	—
1/2	0.9	4.8	0.2	19.3	—
1/4	0.9	18.3	0.8	22.3	—
1/8	7.8	21.7	10.8	22.6	—
1/16	16.9	22.6	20.6	22.9	—
∞	—	—	—	—	25.3

In B, PBMC and sodium azide-treated PBMC and their supernates were prepared as described in Methods. Tested by trypan blue exclusion, the PBMC in the original suspension were 96% viable. After incubation for 20 h, PBMC treated with sodium azide were 3% viable, and untreated PBMC were 92% viable. The PBMC and their supernates were tested for their capacity to inhibit the activation of HF by ellagic acid as in Table I (see Methods). The protein content of the supernate of untreated cells was 78 μg/ml; no protein was detected in the supernate of treated cells. The HF preparation used had a specific activity of 56 U/mg protein.
* In A, PBMC and cycloheximide-treated PBMC and their supernates were prepared as described in Methods. Tested by trypan blue exclusion, the PBMC in the original suspension were 91% viable. After incubation for 16 h, PBMC treated with cycloheximide were 33% viable, and untreated PBMC were 50% viable. The PBMC and their supernates were tested for their capacity to inhibit the activation of HF by ellagic acid as in Table I (see Methods). The protein content of the supernate of untreated cells was 77 μg/ml, and of cycloheximide-treated cells, 10 μg/ml. The HF preparation used had a specific activity of 58 U/mg protein.

Table IX. Effect of Peripheral Blood Mononuclear Supernates on the Activation of HF by Glass and Sulfatides

| PBMC supernate dilution | Amidolysis | |
	Glass*	Sulfatides[‡]
	nmol p-NA released/ml/h	
Undilute	2.0	1.3
1/2	8.1	1.3
1/4	13.8	12.6
1/8	12.8	27.8
∞	10.9	31.0
∞[§]	0.5	—
Undilute[‖]	11.3	—

* Mixtures of 0.25 ml each of a concentrate of PBMC supernate, diluted serially in DPBS, and HF (58 U/mg protein, 0.05 U/ml) were prepared in 12 × 75 mm polystyrene tubes. Aliquots of 0.2 ml were transferred to 10 × 75 mm glass tubes and incubated at 37°C for 60 min with continual shaking. Then, 0.1 ml DPBS and 1.0 ml HPPAN were added to each tube and incubation was continued for an additional 60 min. The reaction was stopped by addition of 0.3 ml glacial acetic acid and the amount of p-NA that had been released was measured against suitable blanks to which glacial acetic acid had been added before HPPAN.
[‡] In 12 × 75 mm polystyrene tubes, 0.1 ml HF (0.05 U/ml, 56 u/mg protein) and 0.1 ml PBMC supernate, serially diluted in DPBS, were incubated at 37°C for 10 min. Thereafter, 0.05 ml 3 × 10⁻⁵ M bovine brain sulfatides in BS was added, incubation was continued for 60 min, 1.0 ml HPPAN was added and amidolysis was measured as described in the preceding paragraph. The PBMC supernate had been concentrated by ultrafiltration and contained 163 μg protein/ml.
[§] Bovine albumin in BS substituted for HF in the original mixture.
[‖] DPBS substituted for PBMC supernate in the original mixture and 0.1 ml PBMC supernate added to the mixture instead of DPBS after the first 60-min incubation.

Bovine brain sulfatides activate HF (2). The supernates of PBMC inhibited activation of HF by sulfatides, but only at higher protein concentrations than needed to inhibit ellagic acid (Table IX).

Discussion

A remarkable property of peripheral blood, much pondered in the nineteenth century, is that the circulating blood is fluid, yet clots promptly when it is withdrawn from blood vessels and transferred to glass tubes. One mechanism through which this comes about is the transformation of Hageman factor (HF, Factor XII) to an enzymatically active state upon contact with certain negatively charged agents (36). Equally remarkable is that the continual contact of plasma with biologically active blood cells does not initiated the clotting process. The experiments that have been described provide some clues to this puzzle, for suspensions of PBMC, including monocytes, lymphocytes (both T and B cells), neutrophils, and to a lesser extent platelets, all appeared to retard the activation of HF by ellagic acid, an agent that in apparent solution readily converts this clotting factor to its activated form. The supernatant fluids in which these cells were suspended shared this property, as if one or more inhibitors of the activation of HF were eluted from the cells. Indeed, since in this study the cells were necessarily suspended in an aqueous medium, it is possible that all of the results described were due to the presence of these inhibitory substances in the fluid phase.

Experiments were performed to determine whether the inhibitory properties of PBMC, monocytes, lymphocytes, and granulocytes were due to contamination with platelets. Preparing PBMC from the blood of a patient with aplastic anemia did not alter the result, nor did partial reduction of the platelet contamination of preparations of PBMC, lymphocytes, and granulocytes. Moreover, supernates of cultured monocytoid cells, uncontaminated with platelets, inhibited activation of HF by ellagic acid.

The nature of the inhibitory agent or agents eluted from cells was only poorly defined. Several lines of evidence suggest that the inhibitory property was related to protein substances in the supernates of cell suspensions. Thus, the inhibitory activity was heat labile, precipitable by ammonium sulfate, and diminished by treatment of cells or supernate with trypsin, or by incubating cells overnight with cycloheximide, an inhibitor of protein synthesis. That the inhibitory property was of cellular origin was further demonstrated by testing the cytosol and particulate fractions of PBMC, monocytes and lymphocytes, both of which blocked the activation of HF by ellagic acid. No evidence was obtained suggesting that inhibition of the activation of HF was enzymatic, nor could we demonstrate binding of HF to insolubilized PBMC supernates.

The inhibitory properties of PBMC supernates appeared to be relatively specifically directed against the activation of HF. The preparations tested did not inhibit the kaolin-activated partial thromboplastin time or the prothrombin time of normal plasma, and did not inhibit thrombin or the activated forms of Stuart factor (Factor Xa), PTA (Factor XIa) or the first component of complement (CĪ of C1 esterase). At very high concentrations, PBMC supernates partially inhibited the amidolytic properties of human plasmin.

Some years ago, the plasma of patients with Hageman trait or with hereditary deficiency of plasma prekallikrein (Fletcher trait) was found to contain agents that were adsorbed to a glass surface, where they reduced the clot-promoting properties of glass (34, 35). Similar agents were also demonstrated in fractions of normal plasma (24). We were, however, unable to demonstrate that the inhibitory properties of PBMC supernates were adsorbed to glass surfaces, suggesting that the supernates contained an agent or agents different from those found in human plasma. Two specific protein components of plasma that inhibit the activation of HF have been identified, β_2-glycoprotein-I (32) and C1q (33). Although this might suggest that the inhibitory property of blood cells may have been due to the selective adsorption of these plasma agents to the cell surface, this did not appear to be the case, for we were unable to demonstrate their presence in concentrates of PBMC supernates by immunodiffusion studies against specific antisera. Another plasma protein, CĪ inhibitor (CĪ-INH), inhibits activated HF (37), whereas PBMC supernates appeared to inhibit the activation of this clotting factor; the presence of CĪ-INH in PBMC supernates could not be demonstrated either functionally or by immunodiffusion against specific antiserum.

Cell walls are rich in proteoglycans. It is unlikely that the presence of these substances in PBMC supernates was responsible for their inhibitory effects, since proteoglycans and their constituent glycosaminoglycans are activators, rather than inhibitors of Hageman factor (38, 39). No uronic acid could be demonstrated in concentrates of PBMC supernates, but this does not exclude the possibility that proteoglycans not containing uronic acid were present.

Because the preparations of PBMC studied were contaminated with platelets, the possibility that the phenomena observed were due to PF4 was of interest. PF4 is known to block activation of the contact system (40). Although we confirmed this observation, we could not demonstrate the presence of PF4 in concentrates of PBMC supernates immunologically, and the addition of heparin to PBMC supernates did not block their inhibitory properties.

The inhibitory activity of PBMC was a property of viable cells, for it was greatly diminished by incubating these cells overnight in sodium azide, a procedure that appeared to kill all but a few cells. Thus, it seems unlikely that the observations recorded were due to the adsorption of plasma proteins to the cell surface.

The question emerged whether the observations reported were due to a specific reaction between cell suspensions or the supernates of these suspensions and ellagic acid. Similar results were obtained, however, when glass or bovine brain sulfatides were used to activate HF.

The supernatants of PBMC, granulocytes, and platelets studied were crude, and presumably contained many different agents. We have not yet been able to assign the inhibitory property of peripheral blood cells to any of their known components, but experiments to this end are in progress. Whether the properties observed are intrinsic to other cells is also not known, but preliminary experiments suggest that similar inhibitory activity is present in the conditioned media in which bovine arterial endothelial cells have been cultured.

The experiments described suggest that intravascular coagulation is impeded by metabolically active cells with which the plasma of peripheral blood comes in contact. Whether these observations reflect significant mechanisms for maintaining the fluidity of circulating blood remains to be elucidated.

Acknowledgments

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