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J Clin Invest. 1977;59(3):549-557. https://doi.org/10.1172/JCI108670.

Research Article

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Leukocyte Procoagulant Activity

ENHANCEMENT OF PRODUCTION IN VITRO BY IgG AND ANTIGEN-ANTIBODY COMPLEXES

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ABSTRACT In a variety of immunologic diseases, fibrin-fibrinogen and immune complexes deposit in areas of tissue damage. However, the mechanisms which initiate fibrin-fibrinogen deposition have not been clarified. We find that the procoagulant activity of human leukocytes is markedly increased after incubation with immunoglobulin and immune complexes. This procoagulant activity is evident after 4–24 h incubation in the presence of as little as 0.1 mg/ml of autologous, isologous, or heterologous IgG. At least three of the four subclasses of IgG myeloma proteins are effective. Experiments with purified rabbit and rat antibodies demonstrate that enhancement of procoagulant activity is significantly greater with soluble antigen-antibody complexes than with immunoglobulin alone. In contrast, insoluble complexes are less effective than immunoglobulin alone. Artifacts due to endotoxin contamination of the IgG preparations were excluded on the basis of the differential sensitivities of immunoglobulin and endotoxin to heat and polymyxin B. Evidence is also presented which shows that enhancement of procoagulant activity involves the production, rather than a simple release, of leukocyte procoagulant activity in vitro.

INTRODUCTION

Proteins of the coagulation system are found in areas of tissue damage in a variety of immunologic diseases. Fibrin-fibrinogen related antigen is present in the rheumatoid joint (1), the rejected kidney allograft (2, 3), and the kidney of lupus nephritis (4, 5). A direct correlation has been found between plasma fibrinopeptide A concentration and disease activity in systemic lupus erythematosus (6). In connective tissue diseases, in acute spontaneous vasculitides (7, 8), and in several types of immunologically-mediated nephritis (9, 11), fibrin-fibrinogen related antigen has been identified at sites of tissue damage. Fibrin-fibrinogen related antigen accompanies immunoglobulin and complement deposits in vascular walls and intercellular spaces. Models of inflammation, such as the Arthus reaction (12), the immune complex disease of rabbits, and the nephritis of the New Zealand Black mouse show fibrin-fibrinogen related antigen deposition and thrombosis as sequelae of parenteral immune complex administration or autoimmune disease.

Leukocyte infiltrates are also present in such lesions, and recent work suggests that these leukocytes are capable of developing potent procoagulant activity. Endotoxin, phyohemagglutinin, and purified protein derivative have all been reported to enhance generation of a thromboplasin-like procoagulant activity in cultures of human leukocytes (13, 14). We have examined the effect of IgG on generation of procoagulant activity in incubates containing human monocytes and lymphocytes. Our observations provide evidence that IgG of all four subclasses markedly stimulates the production of leukocyte procoagulant and that soluble antigen-antibody complexes are significantly more effective than immunoglobulin alone.

METHODS

Isolation and incubation of leukocytes and platelets. Human venous blood was usually collected in commercially obtained (Becton-Dickinson Co., Rutherford, N. J.) evacuated 10-ml glass tubes containing 15 mg potassium EDTA. To exclude artifacts due to the method of blood collection,

This work was published in abstract form. Rothberger, H., T. S. Zimmerman, H. L. Spiegelberg, and J. H. Vaughan. 1976. Enhancement of human mononuclear leukocyte procoagulant activity by IgG and antigen-antibody complexes. *Clin. Res.* **XXIV**: 368A. (Abstr.)

Dr. Zimmerman is the recipient of Research Career Development Award no. HL 70242.

Received for publication 17 June 1976 and in revised form 22 November 1976.

blood was also collected in heparinized (20 U/ml blood) sterile syringes and the results were similar to those with blood collected in evacuated glass tubes. The anticoagulated whole blood was centrifuged at 220 *g* for 10 min at 22°C and buffy coats were removed with a pyrogen-free Pasteur pipette. These buffy coats were pooled and resuspended in 0.9% NaCl-1 mM EDTA adjusted to pH 7.4 and washed twice at 22°C. The cells were then resuspended in 1 vol of 0.9% NaCl-1 mM EDTA (pH 7.4) equivalent to 60% of the original blood volume. 6-ml portions of this suspension were layered on 3 ml Ficoll-Hypaque gradients (15) in 15-ml polyethylene conical tubes and centrifuged for 20 min at 400 *g* at 22°C. Interface cells were removed, pooled, and washed once in 0.9% NaCl (pH 7.4) at 22°C, and twice at 4°C in serum-free RPMI-1640 tissue culture medium (Grand Island Biological Co., Grand Island, N. Y.), and adjusted to 1×10^7 leukocytes/ml in a serum-free synthetic tissue culture medium (RPMI-1640)[1] as determined with a Coulter Counter (Coulter Electronics Inc., Hialeah, Fla.). Leukocyte preparations contained 6–24% monocytes (mean 10%), 76–94% lymphocytes (mean 90%), and 0–3% neutrophils, as determined by light microscopic examination of peroxidase stained smears (16). The ratio of platelets to leukocytes varied from 0.1:1 to 1.0:1 as determined by light microscopy of wet mounts. The leukocyte to erythrocyte ratio was greater than 10:1.

0.1-ml portions of the leukocyte suspension were pipetted into polypropylene tissue culture tubes containing 0.2 ml RPMI-1640. 0.1 ml of a test preparation (protein, endotoxin, or a saline control) was then added, giving a total incubate volume of 0.4 ml and a final leukocyte concentration of 2.5×10^6/ml. Duplicate tubes for each condition were incubated for ¼–24 h at 37°C in a humidified atmosphere of 5% CO_2 and air. Incubates containing leukocytes and human immunoglobulin were usually assayed after 20–24 h. Incubates containing leukocytes and heterologous antigen-antibody complexes were assayed after 4–6 h incubation, when a control incubate with leukocytes and antibody alone produced a clotting time of 75–85 s in the one-stage assay. Control incubates containing leukocytes and antigen, but not antibody, were performed for every experiment with antigen-antibody complexes. At the end of the incubation period, leukocytes were uniformly resuspended and assayed for procoagulant activity by one-stage or two-stage clotting tests.

Platelet-rich plasma was obtained from whole human blood anticoagulated with 1.5 mg/ml of potassium EDTA after centrifugation of the blood collection tubes at 220 *g* for 10 min at 22°C. The platelets were washed twice at 1,000 *g* for 10 min at 22°C in 0.9% NaCl-1 mM EDTA (pH 7.4) containing a protective cushion of 2% autologous erythrocytes, and twice at 4°C in RPMI-1640, also containing erythrocytes. For the final wash, the platelet preparation was centrifuged at 1,000 *g* at 4°C for 45 s, and the platelet-rich supernate was removed. This preparation was free of leukocytes and erythrocytes as determined by light microscopy. The platelets were counted with a Coulter Counter, and added to incubates at the indicated concentrations.

Washing and freeze-thawing of incubates. Incubated leukocytes were washed by the following procedure: tissue culture tubes containing 0.4 ml of incubate were centrifuged at 22°C in an Immufuge (Dade Div., American Hospital Supply Corp., Miami, Fla.) at 500 *g* for 2 min and the upper 0.2 ml of medium was removed. 0.2 ml of fresh medium (80% RPMI-1640, 20% normal saline) was added and the leukocytes were resuspended and centrifuged. This cycle of washing and centrifugation was repeated once. After the final centrifugation, the upper 0.2 ml of wash medium was assayed by the one-stage test. The leukocyte pellet was then resuspended in the lower 0.2 ml of wash medium and similarly assayed. For one experiment, leukocyte preparations were frozen at −20°C after the indicated periods of incubation. 12 h later they were thawed at 22°C and assayed for procoagulant activity.

Sterile techniques. Sterile reagents and plasticware were used in all experiments. Protein solutions were sterilized by filtration with 0.45 μm Millipore filters (Millipore Corp., Bedford, Mass.) or by an ultracentrifugation procedure which clears particles larger than monomeric IgG from the supernate (17). A laminar air flow tissue culture hood was utilized for pipetting procedures. In all experiments bacterial contamination was excluded by light microscopic examination after the period of incubation. The absence of other indicators also excluded bacterial growth (e.g., turbidity and excessive acidity of the culture medium). In the experiments reported here antibiotics were omitted to facilitate the growth and subsequent detection of possible bacterial contaminants. However, in preliminary experiments with isologous IgG, antibiotics were added to the incubation medium (streptomycin 0.1 mg/ml and penicillin 100 U/ml). Results in these preliminary experiments were similar to results reported here.

Purification of immunoglobulins from human sera. IgG was obtained from commercially prepared human Cohn fraction II (ICN Pharmaceuticals, Inc., Life Sciences Group, Cleveland, Ohio) and by DEAE-cellulose chromatography of serum with a 0.015-M sodium-potassium phosphate buffer at pH 8.0. Immunoelectrophoresis of these preparations gave a single precipitin line with horse antihuman serum. Myeloma IgG of slow electrophoretic mobility was isolated from serum by DEAE-cellulose chromatography with 0.01 M sodium-potassium phosphate buffer at pH 8.0. IgG myeloma proteins of fast γ or β electrophoretic mobility were isolated by Pevikon block electrophoresis (Mercer Consolidated Corp., Yonkers, N. Y.) followed by Sephadex (Pharmacia Fine Chemicals Inc., Piscataway, N. J.) G-200 gel filtration. The class and subclass of myeloma immunoglobulin were determined by double diffusion in agar with specific goat antisera. The preparations were dialyzed in 0.9% NaCl and ultracentrifuged according to the method of Chiller and Weigle (17) to remove aggregates and possible endotoxin contaminants. The uppermost ⅓ of IgG solution was removed and used in experiments on the same day.

Preparation of antibodies, antigen, and antigen-antibody complexes. Antisera to ovalbumin (OA) were prepared in rabbits by injection of OA (Nutritional Biochemicals Corp., Cleveland, Ohio) in complete Freund's adjuvant. Antisera to bovine serum albumin (BSA, Armour Pharmaceuticals Co., Chicago, Ill.) were prepared similarly in rats. Antibodies to these antigens were purified by absorption to antigen which had been covalently linked to Sepharose 6B, eluted with 0.5 M glycine-HCl buffer at pH 2.8, and dialyzed into 0.2 M borate buffer adjusted to pH 7.65. Antigen and antibody preparations were then dialyzed in 0.9% NaCl, ultracentrifuged as above, and utilized for immune complex formation. Rabbit Purified rabbit antiovalbumin antibody (anti-OA) purified in this manner gave a single line with the mobility of IgG upon immunoelectrophoresis with an antibody to rabbit whole serum. Freshly prepared complexes were used for each experiment. Rabbit anti-OA-OA complexes were formed by adding 4 mg/ml of purified

[1] *Abbreviations used in this paper*: anti-BSA, purified rat anti-bovine serum albumin antibody; anti-OA, purified rabbit anti-ovalbumin antibody; BSA, bovine serum albumin; OA, ovalbumin; RPCA, relative procoagulant activity; RPMI-1640, a serum-free synthetic tissue culture medium.

antibody to an equal volume of doubling dilutions of antigen at 0.2–25.6 mg/ml in 0.9% NaCl. Controls containing antibody alone or antigen alone were prepared by adding an appropriate volume of 0.9% NaCl to the antibody and antigen preparations. Immune complexes and controls were incubated for 1 h at 22°C. After a 1-h incubation at 22°C, heavy precipitation of complexes occurred at 20:1 and 10:1 antibody to antigen (weight:weight ratios). Light precipitation occurred at 5:1, trace precipitation occurred at 2.5:1 and 1.25:1, and no precipitation occurred at lesser ratios or in the controls. Rabbit anti-OA was added to leukocyte incubates at 0.5 mg/ml, either as antibody alone, soluble complexes, or suspended insoluble complexes. "Soluble complexes" refers to those complexes formed at antibody to antigen ratios of 1.25:1 to 0.156:1. Rat anti-BSA-BSA complexes were similarly prepared, but the antibody was not ultracentrifuged before use.

Protein concentrations were determined from optical densities at 280 nm, with extinction coefficients E 1 cm/1% of 14.3, 7.35, and 6.67 for IgG, OA, and BSA, respectively.

Preparation of substrates. Whole blood anticoagulated with acid citrate dextrose (18) was used as a source of substrate plasma for one-stage coagulation assays and was obtained from normal donors and patients with Factor VIII-deficiency or Factor IX-deficiency. Factor VII-deficient plasma with less than 1% of normal VII activity was obtained commercially (George King Bio-Medical, Inc., Salem, N. H.). A barium sulfate eluate of normal human serum containing factors VII, IX, X, and traces of thrombin and prothrombin was prepared as previously described by Niemetz and Nossel (19) for use in the two-stage test as a source of Factor VII and X.

One-stage assay of procoagulant activity. 0.1 ml of the resuspended leukocyte incubate was placed into a 12 × 75-mm glass tube in a 37°C water bath. 0.1 ml of 0.025 M $CaCl_2$, followed by 0.1 ml of citrated human plasma were added, and the clotting time was determined. For most experiments, plasma from normal donors was used as the substrate. In experiments in which procoagulant activity was further characterized, citrated plasmas from donors with Factor VII, VIII, or IX deficiency were the substrates used. For every condition, the contents of duplicate incubation tubes were each assayed twice, giving a total of four assays. The mean of all four assays is reported. The results of each tube are also indicated by the brackets in Figs. 1–5.

Two-stage assay of procoagulant activity. A more specific assay for tissue thromboplastin than the one-stage test with normal plasma was performed (20, 21). 0.1 ml of the resuspended leukocyte incubate was added to 0.1 ml of a barium sulfate eluate of normal human serum. This mixture was incubated for 5 min in a 37°C water bath and 0.05 ml of 0.025 M $CaCl_2$ was added. After a 1-min incubation, 0.4 ml of 0.015 M sodium citrate was added and 0.1 ml of the resultant mixture was transferred to another tube containing 0.05 ml of an optimal dilution of inosithin. This was followed by addition of 0.1 ml of clotting Factor VII-X-deficient bovine plasma (lot no. 104C 0064, Sigma Chemical Co., St. Louis, Mo.) and 0.1 ml of 0.025 M $CaCl_2$, and the clotting time was determined.

Heat test for endotoxin contamination. Immunoglobulin and endotoxin preparations were diluted to concentrations appropriate for addition to leukocyte incubates (e.g., 4 × incubate concentration) and pipetted into pyrogen-free conical glass tubes which were placed in a boiling water bath for 30 min. Purified endotoxin was obtained from a commercial source (lipopolysaccharide B, *E. coli* 0555:B5, Difco Laboratories, Detroit, Mich.). Crude endotoxin, generously donated by Dr. David Morrison, was obtained by aqueous EDTA extraction of *E. coli* 0111:B4, as described by Leíve et al. (22). The concentration of crude endotoxin was determined by the colitose assay for dioxyhexoses (22). In initial experiments, heat precipitated protein was resuspended by vigorous pipetting and the entire suspension was added to the cultures. In later experiments, the precipitated protein was pelleted by centrifugation at 700 *g* for 10 min, and the supernate was added to cultures. Results with these two methods were similar.

The procoagulant activity of incubates containing heat denatured IgG was compared to that of incubates with undenatured IgG. If the denatured IgG yielded less procoagulant activity than 1/4 or 1/10 dilution of the undenatured IgG, or the saline control, denaturation was considered to be 75, 90, or 100% complete, respectively. Preparations of heated IgG which were less than 75% denatured by heat were considered to be heat stable.

Polymyxin B test for endotoxin contamination. A 0.05-ml vol of polymyxin B (Aerosporin, Burroughs Wellcome Co., Research Triangle Park, N. C.) at 500 μg/ml was added to 0.95 ml of either IgG or endotoxin at four to six times the culture concentration. Before addition to leukocyte preparations, the IgG and endotoxin preparations were exhaustively dialyzed at 4°C in 0.9% NaCl to remove unbound polymyxin B.

Relative procoagulant activity (RPCA). In determining RPCA, a standard curve converting one-stage clotting time to procoagulant activity was utilized. This curve was derived as follows: leukocytes were incubated in the presence of 0.1–1.0 mg/ml of IgG for 4–20 h and then harvested. Doubling dilutions of the incubation mixture in RPMI were assayed by the one-stage test. Clotting time for this test was then plotted against dilution on log-log paper. The clotting time obtained with undiluted leukocytes was arbitrarily given a value of 100, a 1:1 dilution the value of 50, and so on. Analysis of leukocyte incubates from nine donors revealed a logarithmic relationship between clotting time and dilution through the range of clotting times encountered in most experiments (48–120 s). Since the slopes of the nine reference curves were similar, a standard curve was drawn with the derived mean slope of −0.184 (SD 0.02). To determine RPCA, the mean of two one-stage clotting times for a given leukocyte incubate containing immunoglobulin or other added protein was located on the standard curve, and the coordinate of procoagulant activity read. The average procoagulant activity of two control incubates without immunoglobulin or other additives was obtained similarly. Procoagulant activity of the test incubate was then divided by activity of the control. This quotient expressed RPCA. Thus, in each experiment the control was assigned an arbitrary RPCA value of 1, and the values of other incubates were designated as multiples of the control. Procoagulant activity of the incubates containing immunoglobulin or other additives was considered to be enhanced if the RPCA value was 2 or greater. In 10 experiments, the mean RPCA of leukocytes incubated for 20 h with 0.1 mg/ml of human Cohn fraction II was 17.7 (range = 7–33).

RESULTS

Immunoglobulin enhancement of RPCA: augmentation by soluble antigen-antibody complex formation and diminution by insoluble complex formation. The effect of immune complex formation on enhancement of leukocyte RPCA was studied by using

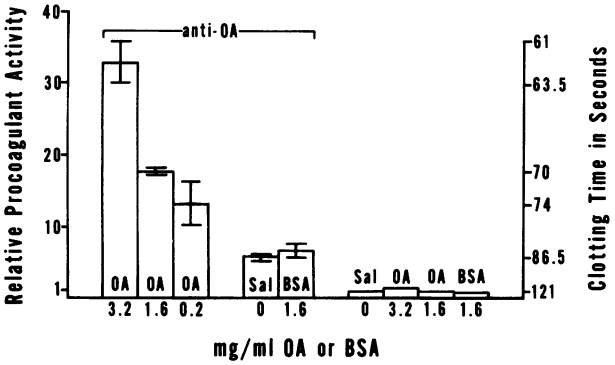

FIGURE 1 Enhancement of leukocyte procoagulant activity by soluble antigen-antibody complexes. One-stage assays of 4-h leukocyte incubates containing the agents indicated on the abscissa are expressed on the ordinate as clotting time and as RPCA. Anti-OA was present at 0.5 mg/ml as indicated by the horizontal bracket (left five bars only). Soluble immune complexes of OA-anti-OA (represented by the group of three bars on left) were more effective than antibody alone in triggering enhancement of RPCA. OA and BSA were no more effective than saline (Sal). Leukocyte preparations were incubated in duplicate tubes. Each tube was assayed twice, giving a total of four assays for each condition. The mean of these four assays is reported. The results of each tube are indicated by the brackets. Clotting times were converted to RPCA by using a standard curve. Control incubates for each experiment (containing saline without added IgG) were assigned an RPCA value of 1, and the activities of other incubates were expressed as multiples of the controls.

solid-phase immunoabsorbed antibody as the source of purified IgG added to incubates. Like other preparations of IgG studied, antibody purified in this manner enhanced RPCA. However, soluble complexes enhanced RPCA significantly more than antibody alone. On the other hand, insoluble complexes enhanced RPCA less than antibody alone. These modulations of the effects of antibody appeared to be solely dependent on specific antigen-antibody interaction, i.e., immune complex formation. Therefore, any significant contribution of RPCA enhancement by unidentified contaminants was highly unlikely. Two antigen-antibody systems, utilizing antibodies from two different animal species and protein antigens from two additional species, gave similar results.

In these experiments, leukocytes were incubated with 0.5 mg/ml of purified rabbit antibody, either as anti-OA alone, as soluble anti-OA-OA complexes, or as insoluble complexes. After 4–6 h, incubates were harvested and assayed for procoagulant activity. Anti-OA alone enhanced RPCA by an average of fivefold (range: two to sixfold) in seven experiments. However, in these same experiments soluble anti-OA-OA complexes increased RPCA additionally by an average of 3.2-fold (range: 2 to 6-fold increase), as compared to anti-OA alone, thus yielding a total average RPCA of 16 (range: 6–32) (Fig. 1). In contrast, in four experiments with suspensions of insoluble complexes (also containing 0.5 mg/ml anti-OA), RPCA was reduced to an average of 47% of the value with 0.5 mg/ml of anti-OA alone (range: 42–60%) (Fig. 2). Controls demonstrated that enhancement or reduction of RPCA after the formation of soluble or insoluble complexes with OA was not the result of an independent effect of OA, nor of a nonspecific response to additional protein. OA alone, incubated with leukocytes in the same amounts and for the same times used with soluble and insoluble complexes (0.025 mg/ml to 3.2 mg/ml, 4–6 h) had no more effect than saline (without anti-OA) (Fig. 1).

Further evidence that OA was acting by promoting the formation of immune complexes was provided by demonstrating that BSA had no effect when added to anti-OA (Fig. 1). However, when BSA was used as an antigen, it increased the ability of purified rat anti-BSA to enhance RPCA. Purified anti-BSA at 0.5 mg/ml enhanced RPCA fivefold in 4-h incubates. When anti-BSA was preincubated with BSA to form soluble complexes, enhancement of RPCA increased an additional 2.2-fold yielding a total RPCA of 11. BSA alone in incubates had no more effect than saline.

Enhancement of RPCA by isologous and autologous IgG: exclusion of endotoxin artifacts with heat and polymyxin B. To examine the effect of human immunoglobulin on RPCA enhancement, several different IgG preparations were studied. Human IgG derived from multiple donors and pooled (Commercial Cohn fraction II Mann Research Laboratories, Inc.,

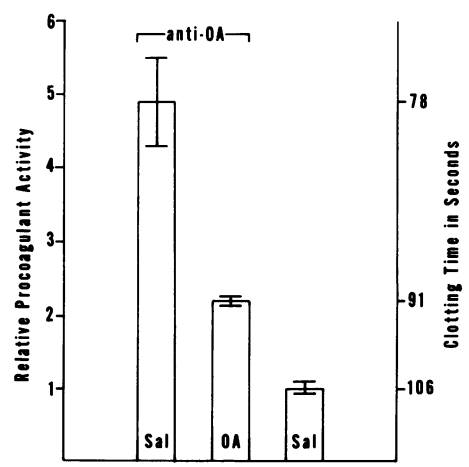

FIGURE 2 Reduction in leukocyte procoagulant stimulating effect of antibody by the formation of insoluble antigen-antibody complexes. One-stage assays of 4-h leukocyte incubates demonstrate that a suspension of insoluble OA-anti-OA complexes formed at 10:1 antibody to antigen ratio (middle bar) yields less RPCA enhancement than antibody alone in saline (Sal) (left bar).

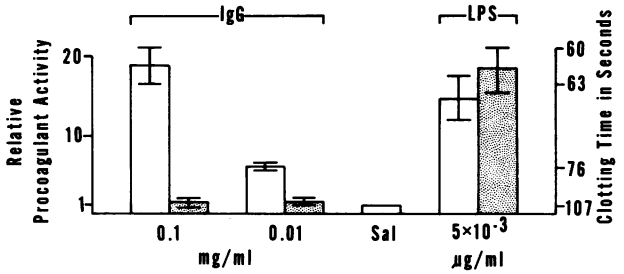

FIGURE 3 Enhancement of leukocyte procoagulant activity by autologous IgG: differential effect of heating on IgG and endotoxin. One-stage assays of 20-h leukocyte incubates containing IgG or purified endotoxin (LPS) at the concentrations indicated on the abscissa are expressed as RPCA and clotting time. The stippled bars indicate results with IgG or endotoxin heated at 99°C for 30 min. The open bars indicate results with nonheated IgG and endotoxin.

New York) and unpooled autologous and isologous IgG, isolated by DEAE chromatography, all enhanced RPCA. The differential sensitivities of these immunoglobulins and endotoxin to heat and to polymyxin B were then examined.

The pooled IgG and three of five unpooled IgG preparations were 75–100% inactivated by heat. In contrast, the ability of endotoxin to enhance RPCA was unchanged by heat (Fig. 3). RPCA enhancement with immunoglobulin was unchanged after polymyxin B addition. Yet, RPCA enhancement with endotoxin was greatly diminished or abolished after the addition of polymyxin B. Preparations of heat stable IgG at 0.5 mg/ml, and purified endotoxin at 10^{-2} μg/ml, yielded 13-fold RPCA enhancement in 20-h leukocyte incubates. Polymyxin B treated IgG yielded 12-fold RPCA enhancement, whereas polymyxin B treated purified endotoxin yielded only 1.6-fold enhancement. Since the purification process can alter the sensitivity of endotoxin to polymyxin B (23), crude endotoxin, extracted from viable *E. Scherichia coli* in an aqueous buffer, was also treated. Crude endotoxin at 0.56×10^{-3} and 0.56×10^{-5} μg/ml enhanced RPCA 7.9-fold and 2.7-fold, respectively. In contrast, after these preparations were treated with polymyxin B, they yielded only 2.7-fold and 1.0-fold enhancement.

Immunoglobulin enhancement of RPCA: dependence on IgG concentration and duration of incubation. To examine the relationship between IgG concentration, duration of incubation, and RPCA enhancement, leukocytes were incubated with different concentrations of IgG and assayed for RPCA after ¼, 4, 7 and 20, and 48-h incubation periods. These experiments demonstrated that RPCA enhancement was IgG concentration-dependent and incubation duration-dependent (Fig. 4). A small but measurable amount of procoagulant activity was also consistently generated in control leukocyte incubates lacking immunoglobulin (Fig. 4). RPCA enhancement with IgG was not merely due to an effect on leukocyte viability or recovery since after 4 h of incubation, there was no loss of viability or recovery from the incubates containing either IgG or saline (99% exclusion of 0.2% trypan blue and 2.4×10^6 leukocytes/ml recovery at 0 and 4 h). Results after 20 h of incubation were similar.

Evidence that a leukocyte-bound procoagulant material is produced in vitro. Experiments in which leukocyte contents were released by freeze-thaw lysis provide evidence that RPCA enhancement is not simply due to release of preformed intracellular procoagulant. Rather, these results suggest that in the presence of IgG, procoagulant material is produced in vitro. Lysates of unincubated leukocytes had no procoagulant activity. Leukocytes incubated without IgG showed only small amounts of procoagulant activity after lysis, equivalent to that of intact cells. In contrast, leukocytes incubated with IgG showed even greater activity after lysis than the corresponding unlysed preparations, and considerably more activity than lysed preparations not containing IgG (Table I). Procoagulant production required IgG interaction with intact leukocytes during the period of incubation. When leukocytes were lysed before incubation

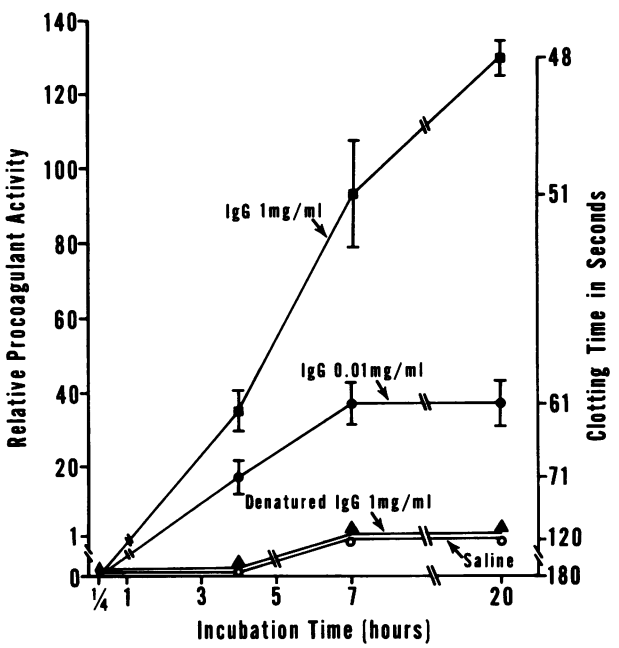

FIGURE 4 Dependence of leukocyte procoagulant activity on duration of incubation and concentration of IgG. Leukocytes were incubated for varying periods with the concentrations of IgG (human Cohn fraction II) indicated in the graph. Heat denatured IgG gave the same result as a saline control without IgG, and demonstrated the absence of endotoxin artifacts in this experiment. No additional increase in RPCA enhancement was observed when incubation was extended to 48 h.

TABLE I

Evidence that a Leukocyte-Bound Procoagulant Material is Produced In Vitro in 5½-h Incubates

Incubate assayed	Incubate contents and clotting time in seconds	
	Saline (control)	IgG
		0.5 mg/ml
Unlysed leukocytes	103	71
Leukocytes incubated and then lysed	116	49
Leukocytes lysed and then incubated	138	142
Leukocyte pellet	111	84
Leukocyte-free supernate	123	128
RPMI-1640 medium	142	142

After incubation with IgG (human Cohn fraction II) or saline, unlysed leukocytes, lysates, and leukocyte-free medium were assayed by the one-stage test. After centrifugation of unlysed incubates (500 *g*, 2 min, 22°C), leukocyte-free supernates and the corresponding leukocyte pellets resuspended in 0.4 ml of medium were assayed separately. Data are reported as described in the Methods section.

and subsequently incubated with IgG for 5½ h, there was no increase of procoagulant activity. Similarly, when leukocytes were incubated without IgG, the addition of IgG immediately before assay had no effect.

Experiments were also performed in which incubates were divided into leukocyte-free supernates and leukocyte pellets by centrifugation. These experiments show that the enhanced procoagulant activity is restricted to the leukocyte pellets and that IgG stimulated activity does not diffuse into the supernate during incubation. When IgG was not present, supernates had more activity than background (medium alone), though not as much as the corresponding unstimulated leukocyte pellets. Incubation with IgG resulted in enhanced activity of the pellets but not of the supernates (Table I). During the washing of pellets procoagulant activity was released into the leukocyte-free wash medium (Table I). These results suggest that procoagulant activity is bound to leukocytes in 5½-h incubates, but is dissociated from intact cells by washing.

Demonstration that myeloma IgG of all four subclasses enhance RPCA. Experiments with monoclonal IgG proteins demonstrated RPCA enhancement with all four subclasses of IgG. By utilizing the standard heat treatment test, the possibility of artifactual RPCA enhancement due to endotoxin was excluded in samples of IgG_1, IgG_2, and IgG_3, since more than 90% of the RPCA enhancement with these samples was abolished by heat. However, heat did not diminish the activity in two specimens of IgG_4 (Fig. 5). Neither of these IgG_4 preparations was available in sufficient quantity to perform the polymyxin B test.

Enhanced tissue thromboplastin-like activity in leukocyte incubates containing IgG. The procoagulant generated in these experiments was characterized by using a two-stage assay for thromboplastin. In each of two experiments 20-h incubates containing 1 mg/ml of IgG (Table II), had markedly enhanced thromboplastin-like activity as compared to incubates without IgG. Assays with Factor VIII, IX, and VII-deficient substrate plasmas also suggested that thromboplastin activity in leukocyte incubates was induced by IgG. After leukocyte incubation, clotting times were

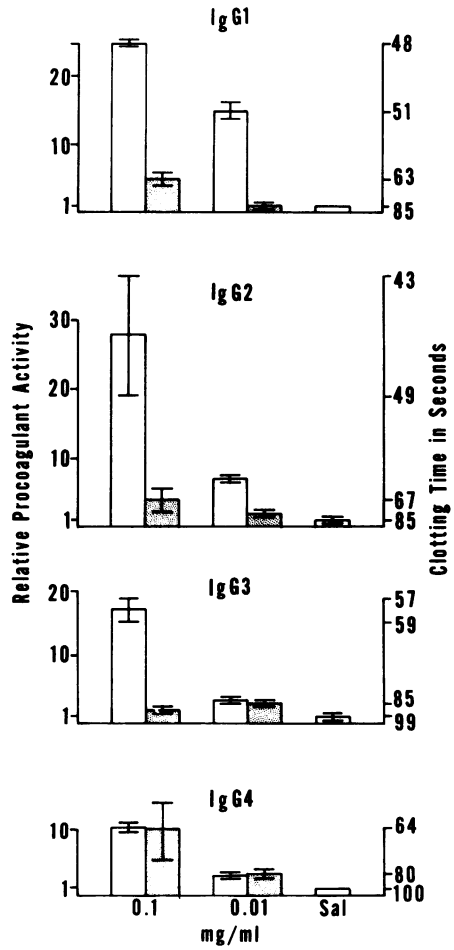

FIGURE 5 Enhancement of leukocyte procoagulant activity by myeloma IgG of all four subclasses. One-stage assays of 20-h incubates containing myeloma containing myeloma proteins are expressed as in Fig. 1. The absence of endotoxin contamination is demonstrated by the heat lability of IgG_1, IgG_2, and IgG_3. The two preparations of IgG_4 were heat stable.

TABLE II
*Enhanced Thromboplastin-Like Activity in 20-h Leukocyte Incubates Containing IgG**

Incubation tube contents		Clotting time in seconds	
Leukocytes	IgG‡	One-stage assay	Two-stage assay
2.5 × 10⁶/ml	*1 mg/ml*		
+	+	46	55
+	±§	115	128
+	−	116	135
−	+	180	177

* Results of one of two similar experiments are given.
‡ Human Cohn fraction II.
§ Human Cohn fraction II denatured by heating at 99°C for 30 min.

shortened to a far greater degree in Factor VIII and Factor IX-deficient plasmas than in Factor VII-deficient plasma (Table III). Nevertheless, some shortening of Factor VII-deficient plasma clotting times was seen in each of five experiments, suggesting the possibility that additional procoagulants were being generated (Table III). Leukocyte preparations containing IgG failed to show any shortening of clotting times when assayed with these deficient plasmas before incubation.

Lack of procoagulant activity in incubated platelets. Since platelets were always present in the leukocyte incubates, and immune complexes induce the release reaction in human platelets, an experiment was carried out to demonstrate that the procoagulant measured by the one-stage test was not produced by platelets alone. Platelets at 2.5×10^6/ml (and free of demonstrable leukocytes) were incubated with IgG at 0.5 mg/ml for up to 20 h. No procoagulant activity could be demonstrated by the one-stage assay beyond that seen with incubation medium alone.

DISCUSSION

The experiments reported here demonstrate enhanced generation of a tissue thromboplastin-like procoagulant activity by human leukocytes after incubation in the presence of IgG and antigen-antibody complexes. Enhancement of procoagulant activity is observed with autologous, isologous, and heterologous IgG. Experiments with myeloma proteins indicate that IgG_1, IgG_2, IgG_3, and probably IgG_4 are effective.

This enhanced activity appears to be due to immunoglobulin stimulated production of procoagulant material in vitro and not simply to immunoglobulin triggered release of preformed material. The small amount of activity generated in incubates lacking immunoglobulin was not increased by lytic release of cell contents. In contrast, lysis of incubates containing immunoglobulin resulted in even greater activity than with intact leukocytes. Lerner et al. similarly provided evidence of in vitro leukocyte procoagulant production by demonstrating that protein synthesis inhibitors prevented the appearance of a tissue factor-like activity during experimental thrombosis (24).

The relationship between antigen-antibody complex formation and enhancement of procoagulant activity underscores the specific role of immunoglobulin in this reaction and excludes artifacts due to contaminants such as endotoxin and procoagulant proteins. Highly purified rabbit and rat antibodies, like other IgG, enhance procoagulant activity production. However, the effect of antibody is potentiated by the addition of antigen in concentrations appropriate for soluble antigen-antibody complex formation. In contrast, the effect of antibody is diminished by adding antigen in concentrations appropriate for formation of insoluble complexes. Antigen itself (BSA or OA) had no effect on the generation of procoagulant activity in cultured leukocytes.

Additional attention was given to excluding artifactual procoagulant activity caused by contamination of the human IgG preparations with endotoxin. Unlike purified endotoxin, most preparations of IgG were denatured sufficiently by heating to 99°C for 30 min to abolish or markedly reduce their ability to enhance procoagulant activity in leukocyte incubates. Thus, heat lability of an IgG preparation provided evidence against significant endotoxin contamination. However, some IgG preparations were resistant to heat denaturation. In experiments with these heat stable immunoglobulins, the sensitivity of endotoxin to polymyxin B was utilized to exclude contamination as the cause

TABLE III
Enhanced Procoagulant Activity in 20-h Leukocyte Incubates Containing IgG with One-Stage Assays with Factor VII, VIII, and IX-Deficient Plasmas*

	Incubate contents and clotting time in seconds	
Plasma used in assay	Saline (control)	
		1 mg/ml IgG‡
Normal plasma	134	74
Factor VII-deficient plasma	154	134
Factor VIII-deficient plasma	145	56
Factor IX-deficient plasma	164	75

* Results of a typical experiment are given.
‡ Human Cohn fraction II in saline.

of RPCA enhancement. An EDTA extracted crude endotoxin and a phenol-purified endotoxin were studied before and after the addition of polymyxin B. The crude endotoxin was not subjected to harsh purification procedures with organic solvents, which modify the biologic effects of endotoxin in other systems (24). Therefore, this crude endotoxin more closely resembles the form of endotoxin one would expect if bacterial growth contaminated the aqueous buffers used for IgG preparation. We have found that after the reaction of both crude and purified endotoxins with polymyxin B, procoagulant activity enhancement is abolished or is greatly reduced. In contrast, in the present experiments, polymyxin B did not reduce procoagulant activity enhancement by IgG preparations. These observations indicate that endotoxin artifacts do not account for enhancement of procoagulant activity by the preparations of human IgG which were resistant to heat denaturation.

The ability of polymyxin B to diminish the RPCA enhancing properties of endotoxin is not surprising. The lipid A moiety of endotoxin has recently been shown to be responsible for enhancing procoagulant activity in leukocyte cultures (25, 26). Other studies have shown that polymyxin B interferes with certain lipid A dependent biological effects such as mitogenicity (24).

It is possible that leukocytes interact with the relatively small numbers of platelets contained in the incubates. That such interactions may be relevant is suggested by the work of Niemetz and Marcus (27), who found that enhancement of procoagulant activity in leukocyte incubates with endotoxin is further increased by the addition of platelets and human serum. Indeed, platelets and unidentified serum factors that are intimately associated with leukocytes may be carried into incubates and be involved secondarily in the phenomena reported here. Nevertheless, platelets free of leukocytes did not produce measurable tissue thromboplastin-like activity in this test system after incubation with IgG. Similarly, Lerner et al. were unable to demonstrate enhancement of procoagulant activity in incubates containing leukocyte-free platelets and endotoxin (14).

The procoagulant activity generated with IgG in these experiments has tissue thromboplastin-like properties. In a two-stage assay for thromboplastin, IgG-containing incubates had enhanced procoagulant activity. Additionally, one-stage assays with Factor VIII or IX-deficient plasma indicate that a leukocyte procoagulant is active in the extrinsic, or tissue thromboplastin pathway. However, although assays with Factor VII-deficient plasma produced considerably less activity, some effect was seen consistently. Thus, there is a possibility that procoagulants other than tissue thromboplastin are generated.

Other studies have demonstrated enhanced tissue thromboplastin-like procoagulant activity of leukocyte incubated in the presence of phytohemagglutinin, purified protein derivative, or endotoxin. IgG can bind to populations of these same cells through the Fc receptor of the leukocyte plasma membrane. Like these other materials, IgG induces the release of a broad range of biological mediators from leukocytes and platelets (28). As demonstrated by our experiments, procoagulant production is an additional effect of IgG on leukocytes.

At this time the relevance of our findings to human disease can only be a matter of speculation. Leukocyte procoagulants may contribute to lesions caused by soluble immune complexes. Thrombosis and deposition of fibrin-fibrinogen in areas of vasculitis, synovitis, and nephritis in animal models of immune complex disease, as well as in humans with diseases such as rheumatoid arthritis, lupus erythematosus, and renal allograft rejection are well documented. Activation of clotting, subsequent thrombosis, and fibrin deposition in these lesions may, in part, result from elaboration of tissue thromboplastin by infiltrating leukocytes. In inflammatory areas, where leukocytes and immune complexes are concentrated, the in vitro conditions described here may be approximated.

ACKNOWLEDGMENTS

We gratefully acknowledge the technical assistance of Miss Jean Rose. We wish to thank Mrs. Anna Milne and Mrs. Phyllis Minick for help in preparing the manuscript, and Dr. David Morrison for helpful suggestions.

This work was supported in part by grants nos. AM 05693, AM 07144, HL 15491, HL 16411, AI 11751, and AI 16734 from the National Institutes of Health, and no. 73-253 from the American Heart Association.

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