IMMUNE COMPLEX-INDUCED HUMAN MONOCYTE PROCOAGULANT ACTIVITY

I. A Rapid Unidirectional Lymphocyte-instructed Pathway*

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The coagulation pathways not only serve hemostatic functions but also appear to be important participants in inflammatory responses, including certain immunologically induced tissue lesions. Local deposition of fibrin is characteristically an early and active process in the immunologic lesions of experimental allergic encephalomyelitis (1, 2), acute proliferative glomerulonephritis (3), delayed cutaneous hypersensitivity (4, 5), and the rheumatoid synovium (6). As one possible mechanism for this phenomenon, it has been observed that human lymphoid cells respond in vitro to diverse immunologic stimuli by generation of cellular procoagulant activity (PCA),[1] an effector limb culminating in the generation of fibrin. Whereas C5a (7), lectins (8), bacterial lipopolysaccharide (LPS) (9), and allogeneic cells (10) have all been described to induce a procoagulant response in vitro, only more recently has it been suggested that immune complexes also possess this potential (8, 11). Rothberger et al. (11) observed a small but significant PCA response of human peripheral blood mononuclear cells to aggregated IgG or soluble immune complexes in antigen excess. Prydz et al. (8) have suggested that immune complexes may directly stimulate the monocyte to produce PCA.

Studies of LPS-induced PCA have provided evidence that the human monocyte is the source of PCA (12, 13). It has also been established that in murine splenic cells stimulated by LPS, the macrophage contains the PCA product (14). In addition, lymphocyte collaboration was strictly required for the induction of splenic macrophage PCA. We have recently suggested in a preliminary report (15) that PCA generated by human peripheral blood mononuclear cells (PBM) can be localized to the monocyte and requires lymphocyte collaboration. Edwards and Rickles (12) have presented evidence for a lymphokine-mediated mechanism in generating a PCA response to LPS and lectin, and have suggested that the lymphocyte may play a facilitating but not a requisite role. In contrast, Prydz et al. (13) suggest that human monocytes are autonomous in the procoagulant response to various stimuli.

In the present study, we investigate the cellular pathways by which soluble immune

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[1] *Abbreviations used in this paper:* Ag:Ab, antigen: antibody; FCS, fetal calf serum; HMEM, Hepes-buffered minimal essential medium; HSA, human serum albumin; LPS, bacterial lipopolysaccharide; PBM, peripheral blood mononuclear cells; PCA, procoagulant activity.

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complexes or aggregated IgG induce PCA response in human PBM and provide evidence that lymphocyte collaboration is required for the immune complex-induced production of PCA by monocytes.

Materials and Methods

Cell Isolation and Culture. PBM were isolated from heparinized venous blood, diluted twofold in RPMI 1640 (Flow Laboratories, Inc., Rockville, Md.) containing 100 U/ml penicillin, 50 μg/ml streptomycin, and 2 mM L-glutamine/ml (Grand Island Biological Co., Grand Island, N. Y.) (incomplete medium), and centrifugated over Ficoll (Sigma Chemical Co., St. Louis, Mo.) -Hypaque (Winthrop Laboratories, New York) (density 1.074 g/ml) at 1,400 *g* for 10 min. The interface cells, cytologically 98% mononuclear, were washed twice in incomplete medium and resuspended in RPMI 1640 containing 10% heat-inactivated fetal calf serum (Grand Island Biological Co., Grand Island, N. Y.) (complete medium). More than 98% of the cells were viable as assessed by trypan blue exclusion; <0.2% of the cells were polymorphonuclear leukocytes.

Adherent cells (monocytes) were isolated by incubating PBM at 1×10^6/ml in complete medium in 16-mm plastic tissue culture plates (24; Costar, Data Packaging, Cambridge, Mass.) at 37°C and 6% CO_2 for 72–96 h. Cells were fed at 72 h with fresh complete medium. Nonadherent cells (lymphocytes) were removed by vigorously washing with medium and the adherent cells (monocytes) were detached by incubation at 22°C for 45 min in Puck's Saline A containing 3 mM EDTA and 3% (wt:vol) bovine serum albumin on a gyrotory platform at 150 cycles/min. Lymphocyte recovery was 72 ± 5% and monocyte recovery was 65 ± 4%. The lymphocytes contained <2% esterase-positive cells, whereas the monocytes contained >98% esterase-positive cells. Nonspecific esterase, a marker for monocytes and macrophages, was assayed according to Li et al. (16).

Platelet-poor mononuclear cells were prepared by first centrifuging whole blood at 120 *g* for 15 min and removing the platelet-rich plasma before isolating PBM by Ficoll-Hypaque centrifugation. PBM thus prepared contained <1 platelet per mononuclear cell, as compared with 35–40 platelets per PBM when prepared in the conventional manner.

Platelets were isolated from blood drawn into acid citrate dextrose anticoagulant. The platelet-rich plasma was obtained by centrifuging the blood at 120 *g* for 15 min. The platelets were either washed twice by centrifuging the platelet-rich plasma at 1,200 *g* for 15 min, and resuspended in incomplete medium, or alternatively, the platelet-rich plasma was passed over a Sepharose CL-2B gel filtration column and the platelets were recovered in the void volume. All platelet preparations contained <1 mononuclear cell per 10^5 platelets.

Lymphocyte-conditioned medium was obtained by incubating lymphocytes with antigen: antibody (Ag:Ab) at 37°C for 1 h, washing the cells twice, and resuspending them in fresh medium for an additional 6 h at 37°C. This second medium was then used as lymphocyte-conditioned medium. Cells were incubated at indicated concentrations at 37°C, 6% CO_2 in 16-mm dishes, or 12- × 75-mm polypropylene tubes (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) in the presence or absence of designated stimuli. All additions to cells were sterilized by filtration through 0.22-μm filters (Millipore Corp., Bedford, Mass.) or by ultraviolet radiation, and assayed for freedom from LPS by amebocyte lysate assay (E-toxate; Sigma Chemical Co.) and by heat inactivation.

Proteins. The IgG fraction of goat anti-human serum albumin, adsorbed three times with human Cohn fraction III and clarified, was isolated by precipitation three times at 50% ammonium sulfate saturation, followed by ion-exchange chromatography on DEAE-cellulose (DE-52; Whatman, Inc., Kent, England) in 0.02 M sodium phosphate, pH 7.2, and molecular sieve chromatography on Sephacryl S-200 (Pharmacia Fine Chemicals, Piscataway, N. J.) in the same phosphate buffer.

The IgG antibody was immunochemically purified from the IgG fraction of the antisera by affinity chromatography on human serum albumin (HSA) (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.) conjugated to cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals). Antibody was eluted at 4°C with 1.0 M glycine-HCl, pH 2.8. The protein was dialyzed into 0.02 M sodium phosphate, 0.15 M NaCl, and 0.3% glycine, pH

7.2. The antibody produced a single precipitin line by double diffusion analysis in gel with rabbit anti-goat whole serum (Bio-Rad Laboratories, Richmond, Calif.) and produced a single precipitin against human serum. Glycine was removed by dialysis before experimental use. Anti-HSA:HSA complexes in slight antigen excess were formed by adding purified antibody (3.7 mg/ml) to an equal volume of doubling dilutions of HSA from 25.6 to 0.2 mg/ml. After a 1-h incubation at 22°C, precipitation was read visually. A concentration of HSA twice as high as that producing precipitation, usually 1.6 mg HSA per mg antibody (molar ratio: 3.5:1, Ag:Ab), was judged to be in antigen excess, and was used for incubation with cells. Concentration of Ag:Ab complexes is expressed on the basis of IgG content. To prepare aggregated IgG, the IgG fraction from nonimmune goat serum at 7.5 mg/ml was incubated at 63°C for 30 min, then centrifuged at 3,000 *g* for 30 min and the soluble aggregates were used. The IgG concentration was determined from absorbance at 280 nm using $E_{280}^{1\%} = 14.3$.

Bacterial LPS and Assay. LPS was isolated from *Escherichia coli* 0111:B4 by the butanol method and was kindly provided by Dr. David Morrison (Emory University, Atlanta, Ga.). The potential for contamination of stimulatory proteins by endotoxin was monitored by a heat-inactivation assay as previously described (17). Heating-aggregated IgG or Ag:Ab solutions to 99°C for 30 min resulted in denaturation and precipitation of the antibody proteins, although endotoxin was not destroyed (18). As such, Ag:Ab or aggregated IgG heated to 99°C for 30 min and added to PBM for 6 h were unable to induce increased PCA from PBM; however, added endotoxin (*E. coli* 0111:B4) retained the ability to induce increased PCA from PBM. All stimulatory proteins and media were found to contain <2 ng LPS per mg of antibody protein.

PCA Assay. Samples were assayed for PCA by reference to their ability to accelerate the spontaneous clotting of recalcified normal human citrated platelet-poor plasma in a one-stage clotting assay (14, 17). The assay consisted of 0.1 ml of sample added to 0.1 ml of normal citrated human plasma in a 12- × 75-mm glass tube, after which 0.1 ml of 0.025 M $CaCl_2$ was added to start the reaction. The time(s) to form a visible clot with constant rocking at 37°C was observed and converted to milliunits of PCA by reference to a standard curve derived from rabbit brain thromboplastin standard (Dade Div., American Hospital Supply Corp., Miami, Fla.) at 36 mg dry mass/ml, which was assigned a value of 100,000 mU. 10-fold serial dilutions were used to produce a standard log-log plot, which was linear from 1 to 10,000 mU and had a precision of 7.6% (coefficient of variation). To assay the expression of PCA on viable cells, 0.1 ml washed cells suspended in RPMI 1640 were assayed. To determine the total content of PCA within the cells, the washed cells in 0.5 ml of RPMI 1640 were first subjected to three cycles of freeze-thawing at −70° and 37°C, followed by sonication (model A-140 sonicator; Heat Systems-Ultrasonics, Inc., Plainview, N. Y.) with two 10-s bursts at setting 3 with ice cooling, and assayed as above. Identification of the type of PCA was done by assay in known coagulation factor-deficient plasmas (George King, Overland Park, Kans.) and RPMI 1640 alone, with or without Ag:Ab or aggregated IgG, were devoid of PCA (0.6 mU PCA/ml containing 1 mg IgG). Data are presented as mean ± standard deviation. Stimulation with Ag:Ab or aggregated IgG yielded almost identical cellular PCA values. As such, the data have been combined.

PCA Plaque Assay. For direct cytologic localization of PCA to individual viable cells, cell suspensions were incubated on glass microscope slides at 37°C for 2 h in complete medium in 6% CO_2. The medium was carefully removed without displacing the cells, and the cells were overlaid with plasma-agarose prepared from three volumes of 2% agarose (Indubiose A-37; L'Industire Biologique, Francaise, S.A.) in Hepes-buffered minimum essential medium, (HMEM) (Flow Laboratories) containing 3.67 g% CaCl, and 1 vol of platelet-poor citrated normal human plasma, which was added to the agarose at 38–39°C. This mixture (1.0–1.5 ml) was immediately poured on the slide to overlay the cells and allowed to gel at 37°C during a 10-min reaction interval. The reaction was terminated by placing the slides in cold HMEM containing 10 U heparin/ml and washed for 16–20 h. The slides were fixed and stained for nonspecific esterase and counterstained with methyl green. Alternatively, other stains such as Giemsa have been used. For assays of total cellular PCA content, the cells were centrifuged onto the slides in a cytocentrifuge, fixed for 45 s in acetone, and dried. The agarose-plasma overlay was applied as described above and after the coagulation reaction was stained for nonspecific esterase. The presence of PCA was clearly indicated by the formation of pericellular strands of fibrin, which contrasted with the red cytoplasmic reaction for nonspecific esterase.

Results

Freshly isolated human PBM contain and express low levels of PCA; however, basal activity increases in vitro to a relatively constant control concentration within 4–6 h in the absence of known exogenous stimuli and in the absence of detectable LPS. The PCA on the surface of viable cells approximated 20% of the total PCA content of disrupted cells (Table I). PBM were separated by adherence for 72 h into lymphocytes and monocytes, and each fraction contained <1–2% of the other cell class by reference to diffuse cytoplasmic nonspecific esterase. From 90 to 93% of the PCA associated with the unfractionated PBM was recovered with the isolated monocytes (Table I). When soluble Ag:Ab complexes or aggregated IgG were added to isolated lymphocytes or monocytes there was no increase in PCA. However, when either stimulus was added to PBM, a 5-fold increase in total PCA content and a 3.8-fold increase in viable expression of PCA as compared with control incubated cells was observed (Table I; Fig. 1). This represented a 30-fold and 22.8-fold higher PCA, respectively, than the basal activity of freshly isolated PBM not subjected to in vitro cultivation.

Kinetics of PCA Induction by Ag:Ab Complexes and Aggregated IgG. Incubation of PBM with either Ag:Ab complexes or aggregated IgG at an arbitrarily selected dose of 90 μg/ml induced progressive increases in cellular PCA over identical time-courses. A representative set of experiments is shown in Fig. 1. A slight increase in PCA of stimulated vs. control cells was evident by 1 h. At 4 h, the PCA response was half maximal, and became maximal at 6 h with only a slight decay of activity over the next 30 h. Cells incubated in the absence of Ag:Ab (control cultures) exhibit a slight increase in PCA with time. The time-course of this increase paralleled the Ag:Ab-induced PCA increase; however, the magnitude of the spontaneous increase in the control cultures was much less.

Dose-Dependence of PCA Response. To identify the threshold for stimulation of PCA and to determine whether there is a dose-dependent effect of the stimulus, increasing

TABLE I

PCA of Human Lymphocytes and Monocytes from Control Culture and after Ag:Ab Exposure

Peripheral blood cell population*	Ag:Ab or aggregated IgG μg/ml	Intact viable cells		Cell homogenates	
		Clotting time	mU PCA/10^6 Cells	Clotting time	mU PCA/10^6 cells
		s		*s*	
PBM	0	63 ± 6	85 ± 11	40 ± 3	495 ± 26
PBM	90	44 ± 4	327 ± 20	28 ± 1	2,040 ± 100
Lymphocytes‡	0	89 ± 11	22 ± 2	66 ± 5	72 ± 14
Lymphocytes	90	92 ± 13	19 ± 3	65 ± 3	72 ± 9
Monocytes§	0	37 ± 2	565 ± 41	25 ± 2	3,180 ± 288
Monocytes	90	39 ± 3	520 ± 36	25 ± 1	3,200 ± 172

* Cells incubated with or without Ag:Ab or aggregated IgG for 6 h, washed, and assayed for viable expression of PCA; or subjected to freeze-thaw and sonication as in Materials and Methods, and assayed for total cellular content of PCA.

‡ >98% esterase negative.

§ >98% esterase positive.

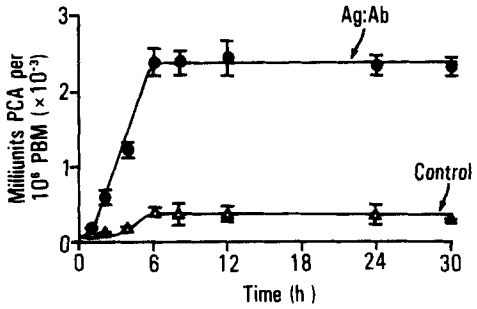

FIG. 1. Induction of PCA in human PBM by Ag:Ab complexes or aggregated IgG. 1×10^6 PBM/ml were incubated in the presence (●) or absence (△) of 90 μg/ml of Ag:Ab of aggregated IgG at 37°C. At various times, cultures were assayed for total cellular PCA content.

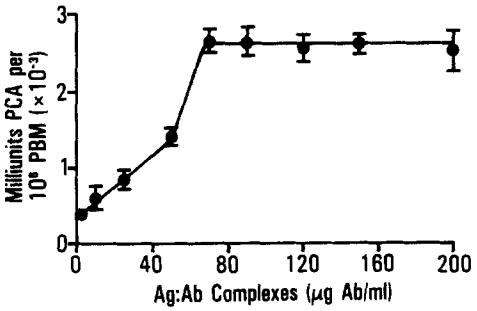

FIG. 2. Induction of cellular PCA by various concentrations of Ag:Ab complexes. 1×10^6 PBM in 1 ml were incubated for 6 h at 37°C with increasing concentrations of Ag:Ab and assayed for total cellular content of PCA.

concentrations of Ag:Ab were incubated with 1×10^6 PBM for 6 h and the cells were assayed for total cellular PCA. There was a 1.5-fold increase over 6 h in control PCA induced by 10 μg Ag:Ab, and a maximal response was obtained with 75 μg/ml, which was relatively constant at higher doses (Fig. 2).

Cellular Origin of Induced PCA. The cellular source of the PCA induced in the PBM preparation was directly examined. PBM were incubated with aggregated IgG or Ag:Ab complexes during the final 12 h of separation by adherence. Lymphocytes and monocytes were then separated and each cell fraction was assayed for total cellular PCA. Lymphocytes showed virtually no increase in PCA (Table II), whereas monocytes from stimulated cultures contained fivefold more PCA than cells from control cultures. Cell death did not account for this difference, as lymphocyte and monocyte viability were virtually identical. Summation of PCA from lymphocytes and monocytes accounted for 112% of the total PCA of whole PBM in control cultures, and 119% of that present in Ag:Ab-stimulated cultures.

To confirm that the monocyte was the cellular source of the PCA, rather than a minor population of cells, a cytologic assay was used to directly visualize cells expressing PCA. Cells (stimulated or control) were incubated on a glass slide to which agarose containing plasma and calcium was carefully overlaid. After a brief incubation, the formation of fibrin around individual cells could be observed. These preparations were stained for nonspecific esterase as a reliable marker for monocytes (21). Cell types and the presence or absence of pericellular fibrin as an index of PCA

TABLE II

PCA of Cell Populations Isolated from Human PBM after Stimulation with Ag:Ab or Aggregated IgG

Cell population*	PCA (mU/10^6 cells)		Stimulated/control
	Control	Ag:Ab or aggregated IGg‡	
Whole mononuclear	455 ± 22	2,050 ± 1,200	4.7
Lymphocytes	72 ± 11	80 ± 4	1.1
Monocytes	3,100 ± 240	16,875 ± 1,430	5.2

* Cells incubated at 1 × 10^6/ml with or without Ag:Ab or aggregated IgG, separated by adherence into lymphocytes and monocytes, then assayed for total cellular content of PCA.

‡ 90 μg/ml.

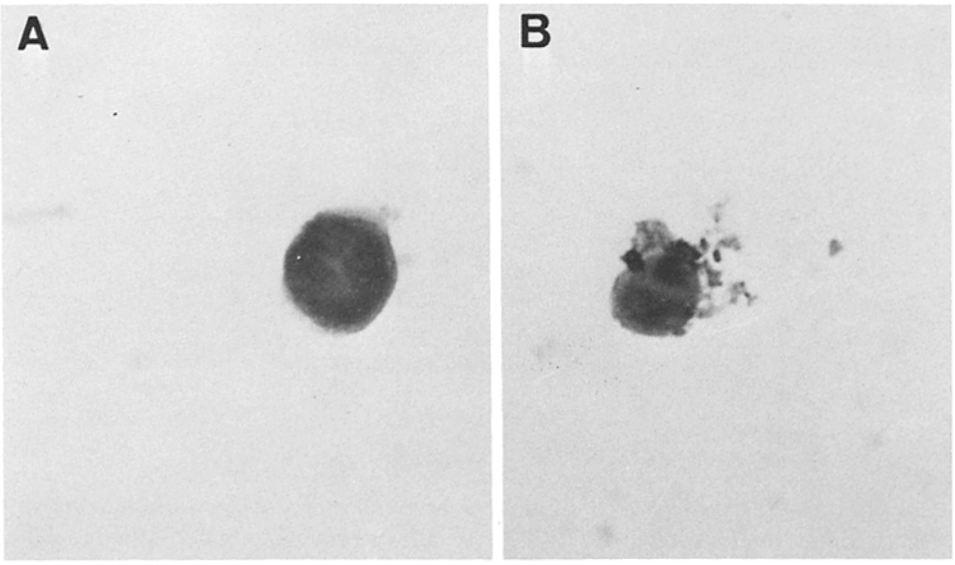

FIG. 3. Cytologic assay for cell type and fibrin deposition among control-incubated and Ag:Ab-incubated cells. Cells were overlaid at 37°C with plasma-$CaCl_2$ containing agarose to allow for fibrin formation, then stained for cellular identification via nonspecific esterase as in Materials and Methods. (A) Esterase-positive cell (monocyte) from control-incubated culture shows no fibrin present. (B) Esterase-positive cell (monocyte) from Ag:Ab-exposed culture with easily identifiable pericellular fibrin formation.

were directly assessed. Upon analysis of cells in unstimulated 6-h control cultures (Fig. 3A), only 2% of lymphocytes had pericellular deposits of fibrin, whereas 15% of the monocytes were positive for PCA (Table III). In a similar analysis of PBM that had been incubated with Ag:Ab or aggregated IgG, 9% of lymphocytelike cells (negative for nonspecific esterase) were PCA positive and 91% of the monocytes were surrounded by fibrin (Fig. 3; Table III). Thus, PCA was predominantly associated with most cells identified as monocytes by simultaneous nonspecific esterase staining and cytology, and was not associated with a minor cell population. The identity of the PCA-positive lymphocytelike cells remains to be resolved.

Lymphocyte Requirement for Generation of Monocyte PCA. Because increased PCA was

TABLE III

Cells Positive for Fibrin Deposition by Cytologic Analysis

Cell population*	Percent of cells positive for fibrin deposition‡	
	Basal	Ag:Ab, 90 μg/ml
Lymphocytes		
Viable	2	9
Fixed	4	13
Monocytes		
Viable	15	91
Fixed	21	100

* Cells incubated with or without Ag:Ab for 6 h, washed, and assayed by cytologic assay in either viable or fixed state as in Materials and Methods.
‡ Mean of 300 cells.

TABLE IV

Cell Populations Required for Amplification of Human Monocyte PCA

Stimulated cell*	Ag:Ab or aggregated IgG, μg/10^6 cells	Collaborating cell	Time	PCA, mU/10^6 cells
			s	
Lymphocyte‡	0	Monocyte	38.2 ± 0.9	600 ± 40
Lymphocyte	100	Monocyte	27.3 ± 0.7	2,415 ± 115
Monocyte§	0	Lymphocyte	37.6 ± 1.6	620 ± 60
Monocyte	100	Lymphocyte	37.7 ± 1.1	625 ± 25

* 1×10^6 stimulated cells incubated with Ag:Ab (100 μg/ml) or sterile saline for 6 h, washed, and added to collaborative cells at a lymphocyte to monocyte ratio of 6:1.
‡ >98% nonspecific esterase negative.
§ >98% nonspecific esterase positive.

seen when PBM, as opposed to lymphocytes or monocytes alone, were incubated with Ag:Ab complexes or aggregated IgG, additional experiments were performed to define the collaborative requirements for induction of monocyte PCA. Lymphocytes and monocytes mixed in physiologic proportion (6:1 lymphocytes:monocytes) in the presence of Ag:Ab complexes or aggregated IgG responded with a 3.6-fold increase in PCA over control cultures (~20-fold over basal 0-h cells). Similarly, lymphocytes that had been incubated with Ag:Ab or aggregated IgG for 6 h, thoroughly washed, and added to monocytes, mediated a 3.7-fold increase in the PCA content of the reisolated monocytes. Incubation of monocytes with Ag:Ab or aggregated IgG, followed by washing and the addition of unstimulated lymphocytes, did not result in PCA induction (Table IV) nor did incubation of monocytes with these stimuli alone (Table I). Indeed, lymphocytes incubated with Ag:Ab or aggregated IgG appeared to represent the sole requirement for induction of an increase in monocyte PCA. This increase in PCA was observed even when non-cell-associated Ag:Ab was removed by washing the lymphocytes before they were added to monocytes. Similarly, erythrocytes incubated with Ag:Ab did not induce increased PCA when added to monocytes (data not shown).

Lymphocyte Requirement for Collaborative Induction of Monocyte PCA. Increasing numbers

of lymphocytes that had been incubated with Ag:Ab were washed and added to a constant number of monocytes (1×10^5 per 16-mm well in 1 ml) and the generation of PCA was monitored (Fig. 4). At ratios of one stimulated lymphocyte per monocyte, no increase in PCA as compared with control cells was observed. The increase of PCA was first observed at two Ag:Ab-stimulated lymphocytes per monocyte and this was maximal at 4:1.

Because standard preparations of PBM contained 35–40 platelets per mononuclear cell, the possible participation of platelets in PCA production was examined. PBM containing 0.2–0.5 platelet per PBM were prepared, and separated into lymphocyte and monocyte fractions. Platelet-poor lymphocytes (<0.5 platelet per lymphocyte) when incubated with Ag:Ab complexes, induced levels of PCA in platelet-poor monocytes to which they were added (Table V) identical to conventional preparations of lymphocytes and monocytes. The presence of platelets in the mononuclear cell cultures did not demonstrably enhance or suppress the PCA response. In addition,

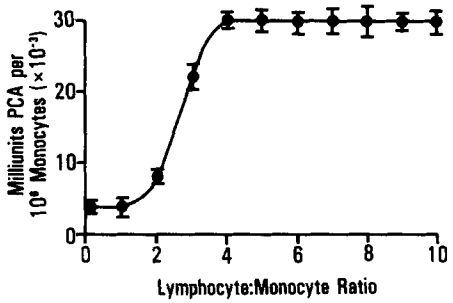

FIG. 4. Lymphocyte collaboration is required for generation of monocyte PCA. PBM were separated by adherence into lymphocyte- and monocyte-enriched fractions, each containing <2% of the other cell type. Lymphocytes (1×10^6/ml) were incubated with 100 μg/ml Ag:Ab complexes or aggregated IgG for 6 h at 37°C, washed twice, and resuspended at various concentrations. Increasing numbers of triggered lymphocytes were then added to 1×10^5 monocytes in a final volume of 1 ml for a further 6 h at 37°C, at which time the cells were assayed for total content of PCA.

TABLE V

Platelets Do Not Substitute for Lymphocytes in Ag:Ab-induced PCA in Human PBM

Cell population added to monocytes*	Ag:Ab	mU total content PCA, 10^6 monocytes
	μg/ml	
None	0	3,450 ± 420
None	100	3,600 ± 270
Lymphocyte‡	0	3,200 ± 300
Lymphocyte	100	17,400 ± 1,450
Lymphocyte plus platelets	0	3,600 ± 400
Lymphocyte plus platelets	100	16,900 ± 1,600
Platelets§	0	3,300 ± 190
Platelets	100	3.400 ± 260

* Cell population incubated with 100 μg/ml Ag:Ab, or sterile saline for 6 h at 37°C, 6% CO_2, washed, and added to 10^5 monocytes for a further 6 h incubation. The monocytes were assayed for total cellular PCA.

‡ 7×10^5 lymphocytes containing <0.4 platelet/lymphocyte.

§ 2×10^7 platelets containing <200 mononuclear cells.

TABLE VI

Intact Viable Lymphocytes Are Required for Mediation of Ag:Ab or Aggregated IgG-induced Monocyte PCA

Monocyte cultured with*	Ag:Ab or aggregated IgG	PCA, (mU/10^6 monocytes)
	μg/ml	
Lymphocytes‡	0	3,200 ± 200
Lymphocytes	100	17,400 ± 1,620
Lymphocyte supernate§	0	3,450 ± 180
Lymphocyte supernate	100	3,600 ± 210
Lymphocyte-conditioned media‖	0	3,320 ± 120
Lymphocyte-conditioned media	100	3,500 ± 200
Disrupted lymphocytes¶	0	3,100 ± 240
Disrupted lymphocytes	100	3,250 ± 110

* 1×10^5 monocytes cultured as indicated. After 6 h, the reisolated monocytes were assayed for their total cell content PCA.

‡ 1×10^6 lymphocytes cultured at 37°C in presence or absence of Ag:Ab for 6 h, washed, and added to 10^5 monocytes.

§ 1×10^6 lymphocytes cultured at 37°C in presence or absence of Ag:Ab for 6 h, medium removed and added to 10^5 monocytes.

‖ 1×10^6 lymphocytes cultured at 37°C in presence or absence of Ag:Ab for 6 h, washed, and incubated for an additional 6 h in fresh culture medium. This second culture medium was removed and added to 10^5 monocytes.

¶ Lymphocytes cultured at 37°C in the presence or absence of Ag:Ab for 6 h, washed, subjected to three cycles of freeze-thaw as in Materials and Methods, and added to the monocytes.

platelets that had been isolated by washing or gel filtration and incubated with Ag:Ab complexes or aggregated IgG did not induce monocyte PCA. Also, such platelets did not modify the observed PCA of monocytes to which they were added (Table V). It should be noted that the platelets were removed from adherent monocytes by washing before the assay of PCA to avoid the direct contribution of any PCA of platelets per se.

Nature of Lymphocyte Collaboration. To investigate the lymphocyte requirement and determine whether lymphocytes per se were required to induce monocyte PCA or whether monocyte induction could be mediated by a soluble product, lymphocytes at 10^6/ml were cultured in the presence of 100 μg of soluble Ag:Ab for 6 h. The supernatant medium was added directly to 10^5 monocytes, incubation was continued for 6 h, and the monocytes were assayed for total cellular PCA. Alternatively, lymphocyte-conditioned medium or lymphocyte homogenate was added to monocyte cultures for 6 h, and the total monocyte PCA was determined. Only when intact viable lymphocytes, which had been incubated with Ag:Ab complexes, were added to monocytes was there induction of monocyte PCA. Neither addition of supernatant medium from the lymphocyte cultures, nor lymphocyte-conditioned medium, nor homogenates of stimulated lymphocytes was able to induce monocyte PCA (Table VI).

Heat Inactivation Studies for LPS. LPS is known to cause an increase in PCA in human PBM (9). To exclude LPS contamination of the stimulatory Ag:Ab or aggregated IgG as a cause of PCA induction, comparative heat inactivation was done (17). By heating aggregated IgG solution to 99°C for 30 min, the protein was

denatured and the ability of the solution to induce PCA in PBM was destroyed in parallel. Whereas IgG is denatured and precipitated, LPS is not (18). As such, LPS heated at 99°C for 30 min was just as stimulatory for PCA as was unheated LPS. By the same token, a solution of Ag:Ab complexes containing added LPS induced PCA. After heating, this solution retained the capacity to induce PCA production, indicating the heat stability of the added LPS. All preparations of Ag:Ab or aggregated IgG were heat labile for induction of PCA excluding LPS at the level of 2 ng/ml Ag:Ab or aggregated IgG. Freedom from LPS contamination at this level has also been confirmed by amebocyte lysate assay.

Nature of the PCA. PBM incubated with Ag:Ab complexes or aggregated IgG were assayed using plasmas from individuals congenitally deficient in a single coagulation factor. The PCA was fully active in Factor XII-deficient plasma (3,600 mU/10^6 cells), Factor VIII-deficient plasma (3,200 mU/10^6 cells), and Factor IX-deficient plasma (3,400 mU/10^6 cells). However, a reduced rate of clotting occurred in plasmas deficient in Factor VII (200 mU/10^6 cells), Factor X (150 mU/10^6 cells), or Factor II (120 mU/10^6). The observations are characteristic of tissue factor-induced coagulation via the extrinsic pathway.

Discussion

As previously described (11) and confirmed in this study, human PBM contain very low basal levels of PCA, which is increased severalfold by exposure to Ag:Ab complexes or aggregated IgG. This increase is observed whether one examines viable cells or disrupts cells to assess the total cellular content of PCA.

The present study provides new and direct evidence that the monocyte is indeed the cell containing most if not virtually all of the basal PCA, and is the source of the PCA induced by incubation with Ag:Ab. Edwards and Rickles (12) have suggested the monocyte origin of cellular PCA using human monocyte-enriched fractions and LPS or phytohemagglutinin as stimuli. However, no rigorous proof has been presented that this is the case for the present system, that the monocyte per se is the responsible cell, or that cellular collaboration is required. The latter seemed unlikely because Ag:Ab and aggregated IgG are known to stimulate phagocytosis and certain other functional characteristics of the activated monocyte or macrophage. Such stimulus-coupled responses have included release of lysosomal hydrolases (19) or enhancement of plasminogen activator secretion (20). Using both cell fractionation and direct cytologic assay, we have directly localized the PCA product to the monocyte after Ag:Ab or aggregated IgG induction. First, when Ag:Ab-stimulated PBM were separated into lymphocytes and monocytes, each population containing <1–2% of the other by nonspecific esterase staining, which is a reliable method of distinguishing between these cells (21), the monocyte fraction contained 96% of the PCA. Second, direct and compelling evidence that monocytes are the source of PCA comes from the cytologic procoagulant assay. This allows direct visualization of cell type by esterase staining and of PCA by the presence of pericellular fibrin. By this method only 9% of lymphocytelike cells were positive for fibrin as compared with 91% of the monocytes from stimulated PBM. It should be noted that before in vitro incubation of the cells there were virtually no PCA-positive viable monocytes, suggesting that this activity may not be displayed by the cell in vivo. These observations establish that indeed the monocyte is the source of PCA and that most if not all monocytes can participate in

the response rather than some minor co-isolated cell population. It is notable that whereas lymphocyte preparations show no increase in PCA following stimulation, cytologic assay did show an increased number of PCA-positive lymphocytelike cells after Ag:Ab stimulation. Evidence from these and other experiments suggests that the cytologic assay may be more sensitive to small amounts of PCA than the one-stage clotting assay, hence the discrepancy might reflect the production of very small amounts of PCA by a few lymphocytes or possibly by monocyte precursors.

The time-course for induction of cellular PCA is extremely rapid, reaching maximal levels within 6 h. This is in contrast to some of the more commonly characterized functions of lymphoid cells, such as lectin-stimulated DNA synthesis or mixed lymphocyte reactions, which require several days to attain a maximum response. Similarly, maximal monocyte/macrophage production of inflammatory mediators such as plasminogen activator (22) and collagenase (23) follows a much more protracted time-course. Evidence for rapid expression of PCA in vivo can be found in lesions such as experimental allergic encephalomyelitis, where perivascular fibrin deposition is one of the earliest histologically identifiable events (1). Whether there is correlation between a very early event, such as PCA production, and later cellular events is under investigation.

The present study indicates that monocytes require unidirectional lymphocyte collaboration in order to manifest an immune complex-induced increase in PCA. Monocytes alone incubated with Ag:Ab or aggregated IgG do not show an increase. Platelets, although possessing Fc receptors (24, 25) and present in PBM populations, did not influence Ag:Ab-induced PCA production by monocytes, nor did they directly substitute for the lymphocyte requirement. Indeed, monocyte PCA was augmented only by incubation with lymphocytes that had been exposed to Ag:Ab or aggregated IgG. The specificity of this stimulus to monocyte PCA is noted by the ability to titrate the amount of PCA produced by adding different proportions of Ag:Ab-triggered lymphocytes, and by the inability of Ag:Ab-incubated monocytes to induce PCA in other monocytes. Somewhat surprising was the inability of Ag:Ab- or aggregated IgG-incubated monocytes to be induced by the subsequent addition of untriggered lymphocytes. Apparently, once the soluble immune complexes have interacted with the monocyte Fc receptor, they cannot subsequently interact with the lymphocyte so as to induce the required lymphocyte "help", or they have been eclipsed from the monocyte surface.

These data provide evidence that the cellular interaction required for Ag:Ab-augmented PCA production is indeed unidirectional in character: lymphocyte → monocyte. This observation contrasts with selected other macrophage responses such as phagocytosis, which is a direct response to relevant stimuli (26), and *Listeria monocytogenes*-induced macrophage-mediated triggering of lymphocytes. In the latter response, the lymphocyte responds by synthesizing and releasing macrophage activating factor, hence forming a circular pathway (27, 28). Furthermore, it appears that intact lymphocytes were required for the collaborative production of monocyte PCA. Neither culture supernates from Ag:Ab-incubated lymphocytes, nor lymphocyte-conditioned medium, nor disrupted lymphocytes were able to substitute for viable lymphocytes. This does not rule out the possibility that a soluble lymphocyte mediator is involved, because the presence of an extremely labile molecule cannot be excluded. Edwards and Rickles (12), have recently reported that supernatant medium

from phytohemagglutinin-stimulated T lymphocyte-enriched (70%) populations could stimulate a partial PCA response when added to monocyte-enriched cell fractions. However, monocytes obtained by such short periods of adherence contained 15% lymphocytes. Whether these lymphocytes had specifically adhered to the monocytes and were able to interact with phytohemagglutinin in the transferred medium and induce PCA production in monocytes is open to question. Their incubation with stimulus was 24 h, compared with 6 h in the present study. This may also contribute to the different findings because there is precedent for viable cells mediating events more rapidly than soluble products (29). However, the possibility should not be dismissed that lectins and Ag:Ab could use different pathways for the induction of lymphoid PCA.

The response to Ag:Ab or aggregated IgG appears saturable in respect to the dose of immune complexes. Moreover, increasing the direct stimulus to monocytes, i.e., increasing the number of stimulated lymphocytes, produced a maximum PCA response at 4:1 lymphocytes to monocytes. This indicates that the ultimate restriction as to magnitude of the response was at the level of the monocyte, not the amount of lymphocyte signal provided.

The PCA produced by monocytes after exposure to Ag:Ab- or aggregated IgG-triggered lymphocytes appeared to be of classical tissue factor type (30). Homogenates of stimulated cells accelerated the clotting of recalcified human plasma deficient in Factor XII, Factor VIII, Factor IX, but not plasma deficient in Factor VII, Factor X, or prothrombin, thus indicating the requirement for these latter factors. PCA was not detected in supernatant medium from monocyte cultures clarified by centrifugation, indicating that cellular assay is sufficient in evaluating this biological response. That this induction results from Ag:Ab or aggregated IgG and not contaminating LPS has been previously validated (11, 17) and was again demonstrated by heat inactivation.

Monocyte participation in initiating thrombosis and fibrinolysis has been implicated before; plasminogen activator is produced by human monocytes, and this response is augmented by the presence of lymphocytes (31). Furthermore, monocytes produce several enzymes that participate at various stages of the inflammationary reaction (19, 22, 23). At such sites, fibrin deposition is frequently associated with immune complex-mediated tissue damage. Based on the present studies, we suggest that monocytes may generate the local PCA required for such local fibrin generation. For example, in experimental immune complex glomerulonephritis, circulating monocyte localization in glomeruli is requisite (32, 33). In addition, the activation of the coagulation pathways and the formation of fibrin may also be required in the pathogenetic sequence (34, 35). It is of interest that the small soluble Ag:Ab complexes generate the greatest increment in PCA (11). Whereas small soluble complexes may be less phlogogenic than the larger complexes that deposit (36, 37), soluble complexes may possess previously unassessed pathogenetic properties, such as inducing the PCA pathway.

It is tempting to speculate on the contribution of lymphoid PCA to phenotypic expression of immune complex disease. For instance, Clarkson et al. (38) have recently hypothesized that the increased rate of atherosclerosis among vasectomized men may be related to the immune complexes present in their serum. Similarly, in the immune complex disease of BxSB hybrid mice, coronary thrombosis and degenerative vascular disease is a prominent associated event (39). It may be necessary to consider whether

elaboration of PCA by monocytes in response to properly stimulated lymphocytes could be a participatory pathway linking the immune system and coagulation process in these diseases.

Summary

It has previously been described that soluble antigen:antibody complexes in antigen excess can induce an increase in the procoagulant activity of human peripheral blood mononuclear cells. It has been proposed that this response may explain the presence of fibrin in immune complex-mediated tissue lesions. In the present study we define cellular participants and their roles in the procoagulant response to soluble immune complexes. Monocytes were shown by cell fractionation and by a direct cytologic assay to be the cell of origin of the procoagulant activity; and virtually all monocytes were able to participate in the response. Monocytes, however, required the presence of lymphocytes to respond. The procoagulant response required cell cooperation, and this collaborative interaction between lymphocytes and monocytes appeared to be unidirectional. Lymphocytes once triggered by immune complexes induced monocytes to synthesize the procoagulant product. Intact viable lymphocytes were required to present instructions to monocytes; no soluble mediator could be found to subserve this function. Indeed, all that appeared necessary to induce monocytes to produce procoagulant activity was an encounter with lymphocytes that had previously been in contact with soluble immune complexes. The optimum cellular ratio for this interaction was four lymphocytes per monocyte, about half the ratio in peripheral blood. The procoagulant response was rapid, reaching a maximum within 6 h after exposure to antigen:antibody complexes. The procoagulant activity was consistent with tissue factor because Factors VII and X and prothrombin were required for clotting of fibrinogen. We propose that this pathway differs from a number of others involving cells of the immune system. Elucidation of the pathway may clarify the role of this lymphocyte-instructed monocyte response in the Shwartzman phenomenon and other thrombohemorrhagic events associated with immune cell function and the formation of immune complexes.

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