MURINE LYMPHOID PROCOAGULANT ACTIVITY INDUCED BY BACTERIAL LIPOPOLYSACCHARIDE AND IMMUNE COMPLEXES IS A MONOCYTE PROTHROMBINASE*

By BRADFORD S. SCHWARTZ,‡ GARY A. LEVY,§ DARYL S. FAIR, AND THOMAS S. EDGINGTON

From the Department of Molecular Immunology, Research Institute of Scripps Clinic, La Jolla, California 92037

Cells of the monocyte-macrophage series can be induced to one or more derivative states by selected biological encounters (1, 2). Directed by the stimulus, diverse responses are observed, among which phagocytosis (3), bactericidal activity (4), and secretion of biologically active products into the extracellular milieu are well documented (5). The immature circulating and migratory members of this series, i.e., monocytes, are immobilized at sites of classical cellular immune reactions and contribute to the features of these and other immunologic tissue lesions. Effector molecules, or monokines, are a diverse and functionally heterogeneous group. Factors such as interleukin 1, colony-stimulating factor, and proteolytic enzymes may be specifically induced and released. These products, which can induce responses in selected cells (6, 7), degrade the extracellular matrix (8, 9), contribute to the characteristics of immune and nonimmune tissue lesions, and proteolytically activate plasma proteins of the coagulation, fibrinolytic, and complement pathways (10, 11).

Tissue lesions mediated by cellular immune responses usually exhibit fibrin deposition. Independently, monocytes have been implicated as major effector cells in these lesions (12, 13). We have recently described the requirements for cellular collaboration in the induction of lymphoid procoagulant activity (PCA)[1] after exposure of cells to bacterial lipopolysaccharide (LPS) or antigen/antibody complexes (Ag/Ab) in murine splenic and peripheral blood mononuclear cells (PBM) (14, 15). Murine peripheral blood and splenic PCA were established as products of monocytes and macrophages, respectively; however, unidirectional instructions from stimulus-triggered lymphocytes were required to rapidly express the monocyte effector molecules in high concentration. Cellular collaboration for the response to LPS require major histocompatibility complex compatibility at I-A (16). Using similar stimuli, induced human PBM initiate the extrinsic plasma coagulation cascade by producing membrane-bound tissue-factor activity (17-21). The significant role that cellular surfaces play in the local deposition of fibrin is exemplified by studies (22) of the platelet membrane as a

focal point for assembly of soluble coagulation proteins into complexes that are kinetically >100,000-fold more efficient in thrombin generation than the soluble proteins alone. Indeed, the formation of activated factor X (Xa) on phospholipid vesicles may depend on a similar membrane surface complex (23). Whether similar membrane assembly mechanisms are involved in the collaboratively induced monocyte PCA responses is not known. In the present report we describe the first exploration of this topic and characterize the inducible murine monocyte PCA. This is a clearly distinctive molecule or set of molecules that differs from the tissue factor activity of induced human monocytes, in that it proteolytically activates prothrombin directly to thrombin or a functional thrombin-like enzyme in the absence of the classical precursors, namely factors VII or X. This appears to represent a new pathway by which selected responses of lymphoid cells may recruit the coagulation pathways in the mediation of inflammatory responses.

Materials and Methods

Mice. C3H male mice of 8–12 wk of age were provided by the Research Institute of Scripps Clinic breeding facility and maintained with water and chow ad lib.

Cells. Peripheral blood was collected from the axillary vein into heparinized capillary tubes (Scientific Products Div., American Hospital Supply Corp., McGaw Park, IL), diluted into 3 vol of incomplete medium (RPMI 1640 containing 100 U/ml penicillin, 50 μg/ml streptomycin, and 2 mM glutamine [Gibco Laboratories, Grand Island Biological Co., Grand Island, NY]), and centrifuged over Ficoll-Hypaque (Sigma Chemical Co., St. Louis, MO and Winthrop Laboratories, Menlo Park, CA, respectively), density 1.074 gm/ml, at 1,400 g for 10 min at 20°C. The cells at the interface were collected, washed twice with incomplete medium, and resuspended at 1 × 10^6 cells/ml in complete medium consisting of 10% heat-inactivated fetal calf serum in incomplete medium. These PBM were >98% mononuclear by cytologic examination of Wright-Giemsa stained preparations, and >99% excluded trypan blue.

Whole PBM were separated by adherence as previously described (15) into lymphocyte-enriched (lymphocyte) and monocyte-enriched (monocyte) populations. 1 ml of 1 × 10^6 PBM in complete medium was incubated in 16-mm flat-bottomed plastic culture wells (24-well; Costar, Data Packaging, Cambridge, MA) for 24 h at 37°C in 6% CO$_2$-air. Nonadherent cells were decanted with the medium and washed. 1 ml of Puck's saline A (Gibco Laboratories) containing 3 mM EDTA and 3% bovine serum albumin (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, CA) was added to the adherent cells, and the plates were rotated on a gyrotory platform at 150 rpm at 22°C for 30 min. The detached monocytes were then recovered, washed, and suspended in complete medium. Recovery of lymphocytes was 83 ± 4%. More than 98% of cells appeared cytologically to be lymphocytes and were negative for nonspecific esterase. Recovery of monocytes was 75 ± 5%; cytologically >98% were of monocyte morphology and positive for nonspecific esterase (24).

In Vitro PCA Response. Cells were incubated at 1 × 10^6 cells/ml at 37°C, 6% CO$_2$ in 16-mm flat bottomed wells in the presence or absence of designated stimuli for periods up to 24 h, as indicated. Complete medium and incomplete medium were equivalent in support of tested functions through 6 h, but for longer cultures complete medium was used. All materials added to cells were first sterilized by filtration through 0.22 μm filters (Millipore Corp., Bedford, MA) or by ultraviolet radiation.

Proteins. Soluble Ag/Ab complexes were prepared from affinity-purified goat antibodies to human albumin as previously described (15). Ag/Ab and culture media were free of LPS contamination (<1 ng/ml) by heat-inactivation (25), limulus amebocyte lysate (E-toxate, Sigma Chemical Co.) and mouse lethality assays (26).

Human factor X and prothrombin were isolated from Cohn Fraction III (kindly provided by Dr. William Brockway, Cutter Laboratories) by sequential chromatography on DEAE-cellulose, heparin-agarose, and homoarginine-agarose as described previously (27, 28). Benzamidine (2 mM) (Aldrich Chemical Co., Milwaukee, WI) and NaN$_3$ (0.02%) were included

throughout the isolations. Both factor X and prothrombin were devoid of detectable contaminants by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (29). Prothrombin was radioiodinated by the glucose oxidase method (Enzymobeads, Bio-Rad Laboratories, Richmond, CA) to a sp act of 4.3–7.2 μCi/μg.

Human factor X was activated following the procedure of DiScipio et al. (30). 20 mg of factor X (5 mg/ml), equilibrated in 0.05 M Tris/HCl, pH 7.8, containing 0.15 M NaCl, 0.02% NaN$_3$ was activated with purified Russell's viper venom (the generous gift of Dr. W. Kisiel and Dr. E. W. Davie) at an enzyme/substrate weight ratio of 1:1,000. The reaction was initiated by adding dry CaCl$_2$ to a final concentration of 5 mM and incubated at 37°C for 20 min. After terminating the reaction by addition of EDTA, the sample was applied to a Sephadex G-100 column (2.5 × 90 cm) equilibrated in PBS (0.01 M phosphate, pH 6.0, 0.14 M NaCl), 1 mM benzamidine, 0.02% NaN$_3$. The activated preparation was rechromatographed over the same column to ensure separation from unactivated factor X and was judged pure by SDS-PAGE using both reduced and unreduced conditions.

Human fibrinogen was isolated from fresh human plasma anticoagulated with acid-citrate-dextrose by differential precipitation according to Doolittle et al. (31). The fibrinogen was exhaustively dialyzed against 0.15 M NaCl, 0.05 M Tris/HCl pH 7.2 and clarified by ultracentrifugation at 100,000 g for 2 h. The purified protein gave the appropriate molecular weight band(s) on SDS-PAGE under both reduced and unreduced conditions and was >97% clottable by α-thrombin. LPS (*Escherichia coli*, 0111:B4, butanol-extracted) was the generous gift of Dr. David Morrison (Emory University, Atlanta, GA).

PCA Assays.   Samples were assayed for PCA by their ability to accelerate the spontaneous clotting time of normal recalcified human platelet-poor plasma as previously described (14, 15). Results were quantitated by comparison to serial dilutions of standard rabbit brain thromboplastin (Dade Div., American Hospital Supply Co., Miami, FL). Human plasmas deficient in specific clotting factors (George King Biological Co., Overland Park, KS) were also used as substrate in the clotting assay in place of normal human plasma. To determine functional prothrombin cleavage, 0.05 ml of fibrinogen (4 mg/ml) was added to 0.05 ml of prothrombin (0.16 mg/ml), and 0.1 ml of cellular sample was added. After addition of 0.1 ml of 25 mM CaCl$_2$, the interval for visual appearance of fibrin at 37°C was determined. Subcellular analyses used the supernatant and pellet from centrifugation at 100,000 g for 1 h of 10^7 monocytes that had been disrupted with a Dounce homogenizer.

Prothrombin Cleavage Assay.   Samples (0.02–0.05-ml) to be tested for prothrombin cleaving activity were added to 0.01 ml of 100 nM ^{125}I-prothrombin and 0.01 ml of 25 mM CaCl$_2$ and incubated at 37°C for 30 min. The reaction was terminated by 1% SDS, 1% EDTA, and the products analyzed by electrophoresis in 10% polyacrylamide, 0.1% SDS in tube gels (28), which were subsequently sliced at 2-mm intervals and analyzed for ^{125}I. Alternatively, reaction products were electrophoresed in 10% polyacrylamide, 0.1% SDS slab gels (32), fixed, dried, and quantitated by autoradiography and soft laser densitometry (Zieneh, Biomedical Instruments, Chicago, IL).

Calcium Requirement and Protease Inhibition Studies.   Controls and samples were assayed for prothrombinase activity in the absence of added calcium and in the presence of 10 mM EDTA. Alternately, cell homogenates (0.04 ml) containing PCA were exposed to serial concentrations of diisopropylfluorophosphate (DFP) for 15 min at 22°C then assayed by the addition of ^{125}I-prothrombin (0.01 ml 100 nM) and CaCl$_2$ (0.01 ml of 25 mM) for an additional 30 min, followed by SDS-PAGE and autoradiographic analysis.

Kinetics and Influence of Cell Number on ^{125}I-Prothrombin Cleavage by Stimulated Monocytes.   For kinetic studies 0.05 ml of cellular homogenates (2 × 10^6 Ag/Ab or LPS-stimulated PBM/ml tris-buffered saline [TBS]) were incubated at 37°C with 0.01 ml ^{125}I-prothrombin (100 nM) (0.045 potential U thrombin), 0.01 ml hirudin (0.2 U) (Sigma Chemical Co.), 0.02 ml CaCl$_2$ (25 mM). At selected intervals, the reaction was stopped by the addition of EDTA and SDS, and ^{125}I-prothrombin cleavage quantitated by SDS-PAGE. Alternately, prothrombin cleavage by homogenates of serial concentrations of monocytes were quantitated as above. Hirudin was used to specifically and rapidly inhibit the thrombin products, thus eliminating reciprocal precursor (prothrombin and intermediates) cleavage by product (thrombin).

Temperature and pH Dependency of the Monocyte Prothrombinase.   1 × 10^6 PBM were incubated

with LPS or Ag/Ab, washed twice with 1.0 ml, and resuspended in 0.5 ml of buffer consisting of 0.14 M NaCl, 0.02 M Hepes at selected pH from 6.0 to 8.5. Cell homogenate (0.04 ml), 25 mM $CaCl_2$ (0.01 ml in buffer of same pH), and ^{125}I-prothrombin (0.01 ml) were combined at 37°C for 30 min. Control incubations contained appropriate pH buffer in place of cell homogenate. The pH of each reaction was determined before the reaction mixtures were analyzed for ^{125}I-prothrombin cleavage by SDS-PAGE and autoradiography. Temperature dependence of the reaction was analyzed at 4, 15, 22, 37, 50, and 70°C.

Analysis for Presence of Zymogen or Inhibitors.   1 × 10^6 LPS- or Ag/Ab-triggered PBM were combined with 0–4 × 10^6 untriggered PBM in a final volume of 1 ml of TBS. The cells were washed twice in 1 ml and resuspended in 0.5 ml of TBS, disrupted as described above, and a 0.025 ml aliquot of homogenate assayed for ^{125}I-prothrombin-cleaving activity.

Plasminogen Activator.   Homogenates of 1–6 × 10^6 PBM in 0.5 ml TBS were analyzed for plasminogen activator by the ^{125}I-fibrin plate assay (10, 33).

Results

Murine PBM in the basal states, i.e., when examined immediately upon isolation, expressed little PCA. In contrast, in vitro cultivation of these cells for 6 h in complete or incomplete medium resulted in a variable but limited nonspecific increase of PCA (50–75 mU/10^6 cells). This was equivalent whether the cells were dispersed over a 2-cm^2 surface area of a plastic culture plate or incubated in a small settled pellet of ~0.1 cm^2 in a 12 × 75 tube, suggesting that attachment is not a significant variable. Incubation of PBM with 100 μg/ml soluble Ag/Ab or 10 μg/ml LPS in complete medium induced a 4-fold increase in PCA as compared with control PBM incubated in the absence of stimuli (Table I), or a 20–24-fold increase relative to cells in the basal state. PCA was evident when intact viable cells (>98% exclude trypan blue) were assayed, but was about fivefold greater when cells were disrupted before assay, indicating that only ~20% of the total cellular PCA was expressed on the surface of viable cells.

Requirements of the cellular PCA for known coagulation factors was investigated by substitution of factor-deficient plasmas for normal plasma in the PCA assay (Table II). Prolongation of the clotting time in plasma deficient in a known factor indicated that the deficient factor was required for the cellular PCA to maximally accelerate coagulation. The quantity of PCA induced by Ag/Ab or LPS was similar, and

Table I

Murine PBM Demonstrate Increased Content of Procoagulant Activity upon Incubation with LPS or Ag/Ab

10^6 PBM incubated with:*	Intact viable cells		Cell homogenate	
	Clotting time‡	PCA	Clotting time‡	PCA
	s	*mU/10^6 cells*	*s*	*mU/10^6 cells*
TBS	58 ± 4	91 ± 17	42 ± 3	388 ± 16
LPS§	46 ± 3	270 ± 21	30 ± 1	1590 ± 31
Ag/Ab‖	47 ± 4	255 ± 26	30 ± 2	1575 ± 56

* 10^6 PBM incubated with indicated stimulus at 37° for 6 h, washed twice in 1 ml TBS, and resuspended in 0.5 ml TBS, then assayed for PCA as described in Materials and Methods.
‡ One-stage clotting assay with normal human plasma.
§ 10 μg/ml.
‖ 100 μg/ml.

TABLE II

Inducible Murine PCA Differentially Supports Clotting In Human Factor-deficient Plasma

Cell stimulus 10⁶ PBM*	NHP‡	Procoagulant activity:(mU) plasma deficient in:§						
		XII	VIII	VII	X	V	II	+II‖
	s	*s*	*s*	*s*	*s*	*s*	*s*	*s*
LPS	31	30	30	30	31	30	>120	31
	(1450)	(1500)	(1520)	(1560)	(1480)	(1520)	(<4)	(1500)
Ag/Ab	30	30	31	30	30	30	>120	31
	(1530)	(1520)	(1420)	(1600)	(1550)	(1530)	(<4)	(1470)

* 10⁶ PBM incubated with indicated stimulus for 6 h at 37°C, washed twice with and resuspended in 0.5 ml TBS, subjected to freeze-thaw sonication, and assayed using the indicated factor-deficient human plasmas.

‡ Normal human plasma.

§ One-stage clotting time with human plasma deficient in the indicated plasma coagulation factor. Values are the mean of three determinations.

‖ Prothrombin-deficient plasma to which 80 μg/ml purified prothrombin was added before assay.

plasmas genetically deficient in factors XII, VIII, VII, X, or V supported the coagulation response as well as normal pooled plasma, indicating that there is no requirement for these factors (Table II). Plasma deficient in prothrombin (factor II) did not clot with the addition of either control or stimulated cells. The addition of physiologic concentrations of purified prothrombin (80 μg/ml) to factor II-deficient plasma restored the cofactor capacity of this deficient plasma.

Prothrombin Activation. The above data suggested the direct use of prothrombin to produce active thrombin or thrombinlike derivatives. To examine the validity of this hypothesis, cells or cellular homogenates were incubated with ^{125}I-prothrombin for 30 min at 37°C, the reactions terminated by addition of SDS and EDTA (the latter to release the prothrombin from the cell membranes), and cleavage analyzed by SDS-PAGE followed by autoradiographic display. In Fig. 1 (lower panel) a single high molecular weight species of intact ^{125}I-prothrombin was observed after incubation with buffer and calcium alone, whereas addition of human factor Xa, the physiologic activator of prothrombin, in the presence of calcium produced cleavage products corresponding to known derivatives of prothrombin. Incubation of ^{125}I-prothrombin with cellular homogenates from stimulated PBM also generated similar products (Fig. 1, top panel), consistent with the direct cleavage of prothrombin by the cellular PCA. On the other hand, after addition of freshly isolated basal PBM to ^{125}I-prothrombin in the presence of calcium, only intact prothrombin was observed (Fig. 1, top panel), indicating that the cellular prothrombinase was not present in an active form.

To determine if the cleavage of prothrombin by monocyte PCA yielded a functional thrombinlike product, cellular homogenates were added to a mixture of purified prothrombin and fibrinogen. Clot formation was monitored after the addition of calcium chloride (8 mM final concentration). Ag/Ab- or LPS-stimulated PBM readily induced the formation of a fibrin clot (Table III). Exclusion of any one of the assay components completely prevented the response, indicating that fibrin formation did not result from a direct conversion of fibrinogen to fibrin by PCA, but that a prothrombin product was required.

Divalent Cation Requirement. PCA-induced clot formation did not proceed in the absence of calcium (Table III). The calcium requirement for cleavage of prothrombin
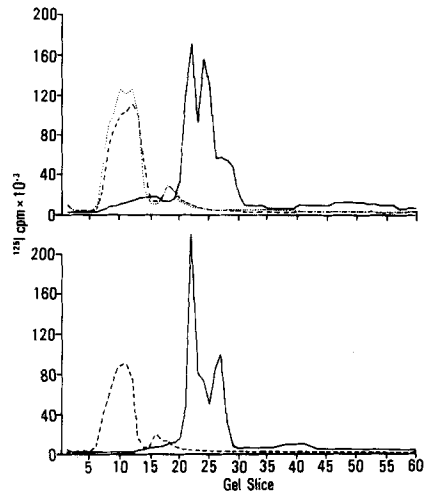
Fig. 1. Cleavage of ^{125}I-prothrombin by human factor Xa and inducible murine prothrombinase. ^{125}I-prothrombin 0.01 ml of (100 nM) was incubated with $CaCl_2$ (0.01 ml 25 mM) and 0.05 ml of test sample for 30 min at 37°C. SDS, β-mercaptoethanol, and EDTA were added, and the samples applied to SDS-10% polyacrylamide tube gels. After electrophoresis, gels were cut into 2-mm slices and counted for radioactivity. Top panel: ^{125}I-prothrombin incubated with: $CaCl_2$ and homogenates from 10^6 unstimulated cells (- - -); EDTA (30 mM) and homogenates from 10^6 Ag/Ab-stimulated cells (·····); $CaCl_2$ and homogenates from 10^6 Ag/Ab-stimulated cells (———). Bottom panel: ^{125}I-prothrombin incubated with: $CaCl_2$ and TBS (- - -); $CaCl_2$ and 150 ng/ml (final concentration) purified human factor Xa (———).

TABLE III

Inducible Monocyte Prothrombinase Forms Functional Thrombin from Prothrombin

10^6 PBM incubated with*	Fibrino-gen 4 mg/ml	Pro-thrombin 100 μg/ml	$CaCl_2$ 25 mM	Clotting time
				s
TBS	+	+	+	>420
LPS or Ag/Ab	+	+	+	46
LPS or Ag/Ab	+	0	+	>420
LPS or Ag/Ab	0	+	+	>420
LPS or Ag/Ab	+	+	0	>420

* 0.1 ml of cell homogenate was tested in the assay mixture, composed of 0.05 ml fibrinogen, 0.05 ml prothrombin, 0.1 ml $CaCl_2$. Final volume was adjusted to 0.3 ml with TBS.

by PCA was examined. Addition of 30 mM EDTA to a reaction mixture of stimulated cell homogenate, ^{125}I-prothrombin, and calcium prevented cleavage (Fig. 1, top panel). This inhibition could be abrogated by the addition of additional calcium (data not shown).

To determine the protease characteristic of the monocyte prothrombinase, cellular homogenates from Ag/Ab- or LPS-stimulated PBM were first incubated with serial concentrations of DFP. After 15 min at 22°C, ^{125}I-prothrombin and calcium were added and incubated for 30 min at 37°C. The effect of DFP on the cleavage of prothrombin is illustrated in Fig. 2. DFP at 2.5 mM or higher prevented the cleavage
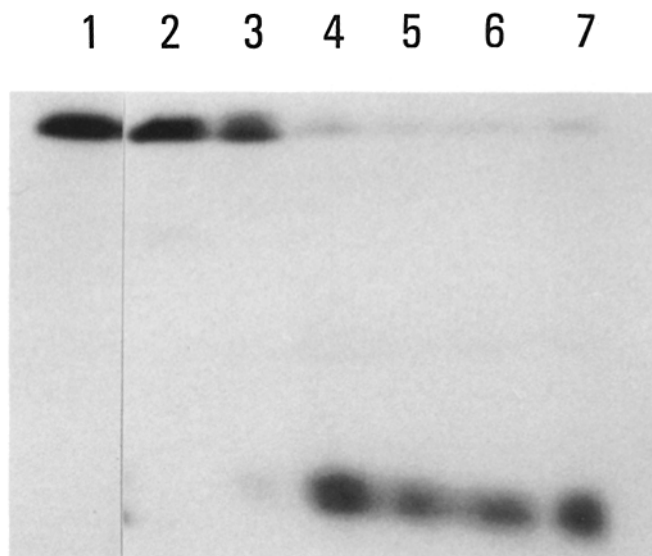
FIG. 2. Inhibition of inducible prothrombinase of murine monocytes by DFP. Homogenates of 1 × 10^6 Ag/Ab-stimulated PBM in 0.5 ml TBS were incubated for 15 min at 22°C with the indicated concentration of DFP. To 0.05 ml of each reaction was added 0.01 ml ^{125}I-prothrombin (100 mM) and 0.01 ml CaCl$_2$ (25 mM) for 30 min at 37°C. SDS and EDTA were then added, and the samples electrophoresed on a SDS-10% polyacrylamide slab gel and displayed by autoradiography. Lane 1: ^{125}I-prothrombin, CaCl$_2$, TBS. Lanes 2–7: ^{125}I-prothrombin, CaCl$_2$, prothrombinase containing cell homogenates that had been incubated with 10, 2.5, 1.0, 0.62, 0.16, and 0.04 mM DFP, respectively.
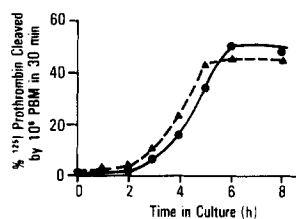

FIG. 3. Appearance of prothrombinase in murine PBM with increasing times of exposure to Ag/Ab or LPS. PBM at 10^6 in 1 ml complete medium were incubated at 37°C for increasing times in the presence of 100 μg/ml soluble Ag/Ab (●) or 10 μg/ml LPS (▲). At the times indicated, cells were washed twice in 1 ml and resuspended in 0.5 ml TBS. After disruption, cellular homogenates were combined with ^{125}I-prothrombin and CaCl$_2$ for 30 min at 37°C, and the reactions analyzed for ^{125}I-prothrombin cleavage by SDS-PAGE and autoradiographic display.

of prothrombin, whereas at 1 mM or less no inhibition was observed. This effect differed from that of factor Xa, because 10 mM DFP was required to inhibit factor Xa in the presence of unstimulated cellular homogenates.

Membrane-associated Monocyte Prothrombinase. The prothrombinase activity of PBM progressively increased between 2 and 6 h after exposure to stimuli. Maximum levels of prothrombinase activity were expressed by PBM after 5 h exposure to LPS, and after 6 h incubation with Ag/Ab (Fig. 3). Simultaneous exposure of cells in culture to both LPS and Ag/Ab did not further increase PCA (1,620 mU/10^6 cells) to levels greater than exposure to either LPS (1,590 mU/10^6 cells) or Ag/Ab (1,650 mU/10^6

cells) alone, indicating a nonadditive response and suggesting a quantitatively limiting step.

We have previously shown (14, 15) cells of the monocyte/macrophage cell lineage to be the origin of collaboratively induced PCA. In the present study, PBM were separated by adherence into monocytes and lymphocytes. Prothrombinase activity was observed in the monocyte population in contrast to its absence in lymphocytes (Table IV). Whereas lymphocytes have been shown to be necessary for induction of murine monocyte PCA by LPS or Ag/Ab (14, 15), the effect of lymphocytes on the expression of monocyte prothrombinase, once it had been induced, has not previously been examined and was tested. Homogenates of 5×10^5 monocytes from Ag/Ab or LPS triggered PBM-induced fibrin formation in reaction mixtures of only prothrombin, fibrinogen, and $CaCl_2$ in 33 ± 2 s in the absence or presence of 0.5, 1, 2, or 4×10^6 lymphocytes, indicating that lymphocytes do not influence the functional activity of this monocyte product once produced.

Partial subcellular localization of the enzymatic activity was carried out by fractionation of 10^7 induced or control monocytes. After disruption and sedimentation of nuclei, the cytosol fraction (1 h at 100,000 g supernatant) was devoid of prothrombin-cleaving activity (<5% ^{125}I-prothrombin cleaved), whereas the membrane/micro-organelle pellet, when resuspended in the original volume of TBS, contained potent prothrombinase activity (>97% ^{125}I-prothrombin cleaved).

The amount of prothrombin-cleaving activity was found to be related in a linear fashion to the number of Ag/Ab or LPS induced monocytes present in the assay. ^{125}I-prothrombin cleavage (Fig. 4) was marginally increased after 30 min at 37°C with

TABLE IV
Prothrombinase Activity of Cell Populations from LPS- or Ag/Ab-stimulated Murine PBM

Stimulated cell population*	Clotting time 10^6 cells		Percent ^{125}I-prothrombin cleaved/10^6 cells‖
	Normal human plasma‡	Purified compo-nents§	
	s/mU	s	
PBM	31/1,420	51	58
Lymphocytes	85/27	420	3
Monocytes	21/8,200	27	98

* 10^6 PBM in 1 ml complete medium incubated with 10 μg/ml LPS or 100 μg/ml Ag/Ab for the final 6 h of separation. After separation, cells were washed twice in 1 ml TBS, resuspended in 0.5 ml TBS, disrupted, and assayed for prothrombin cleaving activity.

‡ One-stage plasma recalcification test using 0.1 ml normal human plasma, 0.1 ml test sample, and 0.1 ml 25 mM $CaCl_2$, combined at 37°C, and the time required for fibrin clot formation recorded.

§ One-stage clotting assay: 0.05 ml fibrinogen (4.0 mg/ml), 0.05 ml prothrombin (160 μg/ml), 0.1 ml test sample, and 0.1 ml $CaCl_2$ were combined at 37°C, and the time required for fibrin clot formation recorded.

‖ Cleavage was determined after 0.01 ml of ^{125}I-prothrombin (100 nM) was incubated with 0.01 ml 25 mM $CaCl_2$ and 0.05 ml test sample at 37° for 30 min. Reactions were stopped by addition of SDS and EDTA, and applied to a 1% SDS-10% polyacrylamide slab gel. After electrophoresis, fixing, and drying, autoradiography was performed and percent cleavage of ^{125}I-prothrombin determined by densitometry.
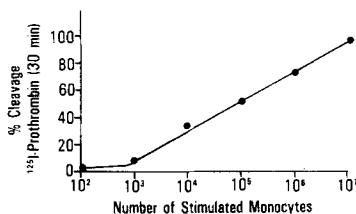
FIG. 4. Cleavage of ^{125}I-prothrombin by differing numbers of prothrombinase-positive murine monocytes. Murine PBM were incubated with Ag/Ab or LPS for 6 h, after which the cells were separated by adherence. Monocytes were disrupted, and 0.05 ml of cell homogenate was combined with 0.01 ml of 100 nM ^{125}I-prothrombin (0.045 potential U of thrombin), 0.2 U hirudin in 0.01 ml, and 0.01 ml of 25 mM CaCl₂ for 30 min at 37°. Reactions were analyzed for ^{125}I-prothrombin cleavage by SDS-PAGE, autoradiography, and soft laser densitometry.
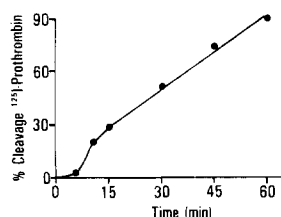

FIG. 5. Kinetics of ^{125}I-prothrombin cleavage by 10^6 murine Ag/Ab- or LPS-stimulated PBM. Homogenates of 10^6 stimulated PBM (0.05 ml) were incubated with 0.01 ml of 100 nM ^{125}I-prothrombin, 0.2 U hirudin in 0.01 ml, and 0.01 ml of 25 mM CaCl₂ at 37°C. At the times indicated, the reactions were stopped by the addition of EDTA and SDS, and the samples subjected to SDS-PAGE and autoradiographic analysis.
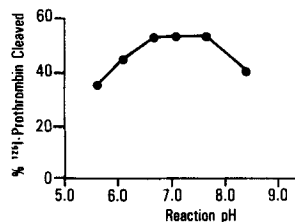

FIG. 6. Influence of pH on proteolytic activity of inducible murine monocyte prothrombinase. Homogenates of 10^6 PBM/ml were prepared in Hepes-buffered saline at pH 6.0, 6.5, 7.0, 7.5, or 8.0. Aliquots (0.04 ml) were incubated for 30 min at 37° with 0.01 ml of 25 mM CaCl₂ (in appropriate pH buffer) and 0.01 ml ^{125}I-prothrombin. At the end of incubation, before addition of SDS and EDTA, the pH of each reaction was confirmed. ^{125}I-prothrombin cleavage was determined by SDS-PAGE and autoradiographic analysis.

homogenate from 1 × 10^3 PCA-positive monocytes. Under identical conditions, a progressive and linear increase in prothrombin cleavage was observed with logarithmically increasing numbers of cells up to 10^7 monocytes. The dose-effect was consistent with a first order reaction.

The kinetics of prothrombin cleavage by 10^6 stimulated PBM were examined in the presence of hirudin to inhibit thrombin- (generated by action of PCA on prothrombin) mediated cleavages of the prothrombin substrate, a secondary in vitro proteolytic consequence of prothrombin activation. PCA-induced PBM were washed, disrupted, and homogenate equivalent to 10^6 cells was incubated with ^{125}I-prothrombin and CaCl₂. At various times, samples were removed, the reaction terminated with
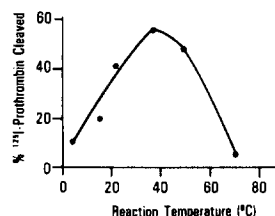
Fig. 7.   Temperature dependence of the inducible murine monocyte prothrombinase. Homogenates of 10^6 stimulated PBM/ml in TBS were prepared; and 0.04 ml aliquots of homogenate, 0.01 ml of 100 nM ^{125}I-prothrombin, and 0.01 ml of 25 mM $CaCl_2$ were incubated for 30 min at 4°, 15°, 22°, 37°, 50°, or 70°C. The reactions were terminated with EDTA and SDS, and analyzed for ^{125}I-prothrombin cleavage by SDS-PAGE and autoradiography.

TABLE V

Murine Monocytes Do Not Possess a Preexisting Inhibitor or Zymogen of
Inducible Prothrombinase Activity

Number of unstimulated PBM added to 1 × 10^6 PBM possessing prothrombinase activity*	Percent ^{125}I-prothrombin cleavage‡	Percent augmentation (+) or inhibition (−) of ^{125}I-prothrombin cleavage
0	46	—
1 × 10^6	43	−6.5
2 × 10^6	50	+8.7
3 × 10^6	43	−6.5
4 × 10^6	48	+4.4

* 1 × 10^6 PBM/ml, which had been incubated with 100 µg Ag/Ab or 10 µg LPS, were shown to possess prothrombinase activity and were combined with varying numbers of basal unstimulated cells in a final volume of 1 ml of TBS for 15 min at 22°C, then disrupted by freeze-thaw sonication before assay for ^{125}I-prothrombin cleavage, as described in Materials and Methods.
‡ Cell homogenate (0.025 ml), ^{125}I-prothrombin (0.01 ml of 100 nM), and $CaCl_2$ (0.01 ml of 25 mM) were incubated at 37°C for 30 min. EDTA and SDS were added, and the samples analyzed for ^{125}I-prothrombin cleavage by SDS-PAGE, autoradiography, and laser densitometry.

SDS and EDTA, and the extent of cleavage characterized by SDS-PAGE (Fig. 5). There was an apparent 5–10 min initial lag period followed by a linear rate of prothrombin cleavage to 60 min, when >90% of the substrate was consumed. This lag period could be eliminated if the number of PCA-induced PBM present in the incubation mixture was increased to 10^7 (data not shown).

The monocyte membrane prothrombinase induced by Ag/Ab or LPS was further analyzed for pH and thermal optima. As seen in Fig. 6, the inducible prothrombinase recovered in cellular homogenates cleaved optimally at pH 6.7–7.6. Maximal cleavage was observed at the thermal optimum of 37°C (Fig. 7).

Inhibitor or Zymogen Forms of the Enzyme.   The possibility that the induction of PCA in murine monocytes results in the inactivation of a coexisting inhibitor, or activation of a zymogen form of the prothrombinase was explored. A constant number (1 × 10^6) of LPS- or Ag/Ab-induced PBM were combined with 0–4 × 10^6 unstimulated PBM in 1.0 ml of TBS. The latter cells serve as a source of putative inhibitor or zymogen. The cell mixtures were disrupted and assayed for prothrombin cleaving activity (Table V). The lack of effect of the added unstimulated cells indicates that they lack

an inhibitor, thus excluding the hypothesis that the increase of PCA after stimulation results from a reduction in concentration of an inhibitor. Similarly, the unstimulated cells would have provided additional zymogen with an increase of activity if the induction event represented the production of an enzyme that cleaved the prothrombin zymogen to active prothrombinase, or if the prothrombinase was autoactivating. Because this was not observed, we conclude that the appearance of prothrombinase activity probably represents synthesis of new enzyme molecules.

Plasminogen-activator Activity. Homogenates of 1, 2, 3, 4, 6, or 8 × 10^6 PBM stimulated with small soluble Ag/Ab (4:1 ratio) for 6 h were prepared in 0.5 ml of TBS and assayed for plasminogen-activator activity. The addition of cellular homogenates of stimulated PBM to the plasminogen and radioiodinated fibrin led to release of no more radioactivity (217 ± 17 CPM) than did wells containing TBS, plasminogen, and ^{125}I-fibrin alone (208 ± 26 cpm).

Discussion

The rapid collaborative PCA response of murine splenic mononuclear cells to LPS has been recently characterized (14). A PCA response is also observed for murine PBM exposed to soluble Ag/Ab or LPS (15). In these studies, the macrophage or monocyte, respectively, was identified as the cellular source of PCA and the collaborative requirement for intact viable lymphocytes was established.

In the present study we describe and characterize this murine monocyte PCA as an inducible prothrombinase, a new pathway of recruitment of the coagulation pathways. In experiments using plasmas deficient in coagulation factors XII, VIII, VII, X, V, and II (prothrombin), only the latter failed to support fibrin formation in the presence of the cellular PCA; addition of purified prothrombin to factor II-deficient plasma restored the ability of this plasma to serve as substrate for the cellular PCA. The inducible PCA thus exerted its effect through neither the classical intrinsic nor extrinsic procoagulatant activation cascades (34), but rather by direct action on prothrombin in the late stage of coagulation.

To substantiate this unanticipated prothrombinase-like activity, prothrombin was incubated with homogenates of stimulated or unstimulated PBM in the presence of calcium, and the products of this reaction were analyzed. ^{125}I-prothrombin, which had been incubated with unstimulated PBM, remained intact by SDS-PAGE. However, the addition of PCA-positive PBM to ^{125}I-prothrombin resulted in prothrombin cleavage to lower-molecular weight products comparable to those resulting from cleavage by purified factor Xa and included thrombin per se or an indistinguishable product. This was not the result of thrombin contamination or secondary cleavage of prothrombin by generated thrombin, because identical cleavage was observed in the presence of hirudin, a rapid and potent inhibitor of thrombin. The thrombinlike product of the PCA action on prothrombin efficiently converted purified fibrinogen to fibrin, whereas PCA positive cellular homogenates did not directly cleave fibrinogen or convert it to fibrin. Thus, the evidence suggests the inducible PCA acts by directly cleaving prothrombin to an active thrombinlike enzyme, if not thrombin per se, capable of inducing the fibrinogen-fibrin transformation.

Monocytes are the source of the induced prothrombinase and require the presence of intact stimulus-triggered lymphocytes for prothrombinase induction. However, once induced, the presence of lymphocytes was without effect on the product.

Prothrombinase was not detectable in 10^7 freshly isolated murine monocytes, but was demonstrable in as few as 10^3 monocytes that had been incubated with lymphocytes and stimulated with soluble Ag/Ab or LPS. This represents at least a 10,000-fold increase in monocyte prothrombinase. The prothrombinase did not appear to result from uptake of serum factors by cells in culture, insofar as full prothrombinase activity was observed in thoroughly washed cells cultured in the absence of serum.

Inducible lymphoid PCA is apparently expressed at the surface of viable peripheral monocytes and splenic macrophages, because viable cells induce coagulation (14, 15). We observed surface expression of prothrombinase activity in the present study as well. In agreement with our previous studies (14, 15), we found by the more definitive prothrombin cleavage assay that surface expression of PCA was no more than 20% of the total cellular content. Partial subcellular fractionation by ultracentrifugation localized the prothrombinase to the crude membrane/small organelle fraction. The active prothrombinase is not released or secreted into the medium. As such, monocyte prothrombinase appeared to be the major form of murine lymphoid PCA previously described (14, 15) for the response to LPS and Ag/Ab. This differs from human lymphoid cells, which respond to the same stimuli by production of tissue factor, the activator of the extrinsic coagulation pathway (35). It should be noted, however, that human monocytes can be induced to produce a prothrombinase by certain subfractions of plasma lipoproteins (28), a response that, although nonimmunologic, also requires lymphocyte collaboration (36).

The inducible murine monocyte prothrombinase has characteristics consistent with a serine protease, i.e., inhibition by DFP. Indeed, the concentration of DFP required for enzyme inhibition was well within the range required by a number of the serine proteases of the coagulation cascade (37). Notably, the monocyte prothrombinase was more sensitive to DFP than factor Xa, the classical prothrombinase. The monocyte prothrombinase required the presence of calcium to cleave prothrombin. However, it is not clear whether this calcium requirement was for the prothrombinase or the prothrombin substrate. Prothrombin must bind calcium via its gamma-carboxyglutamic acid residues to undergo requisite conformational transitions and then bind to phospholipid to render appropriate domains susceptible to proteolysis (38). Whether there are similar types of requirements for the prothrombinase remains to be investigated. The cleavage of prothrombin substrate was linear with time, and cleavage increased linearly in proportion to the log concentration of induced monocytes. Both data suggest a direct first-order proteolytic event exhibiting no cooperative effects.

A number of possible mechanisms might account for the present observations. Triggered lymphocytes could induce monocyte PCA by: (a) inducing synthesis of active prothrombinase; (b) suppressing synthesis of a prothrombinase inhibitor; or (c) induction of a protease that converts a zymogen to the active prothrombinase. Unstimulated cell homogenates were added as a source of hypothetical inhibitor or zymogen to stimulated PBM cell homogenates and did not modify prothrombinase activity. From this we conclude that unstimulated cells do not contain an inhibitor to the prothrombin activator or a zymogen of the prothrombinase analogous to proteases of the coagulation cascade present in plasma in the zymogen form (34, 37). Thus, the most reasonable postulate is that LPS- or Ag/Ab-triggered murine lymphocytes induce I-A-compatible monocytes (16) to synthesize the active prothrombinase.

Mononuclear phagocytes serve an important role in tissue inflammation and repair.

Some macromolecular products of monocytes or macrophages can be assigned roles in inflammatory lesions. Collagenase, inducible by phagocytosis (8), and elastase, also inducible (9), have recognizable functions in inflammation (39). Plasminogen activators (19) are induced by soluble products of stimulated lymphocytes (40) and serve as markers of macrophage activation (2). The inducible monocyte prothrombinase is not a plasminogen activator; but the relationship to some other monocyte neutral proteases has not been explored. The possibility that the monocyte prothrombinase could be factor Xa has been examined for human cells (28), and appears to be incorrect. Limited evidence suggests that murine macrophages cultured in vitro might produce factor X (41). However, the murine monocyte prothrombinase does not appear to be factor Xa based on susceptibility to inhibitors, molecular weight, prothrombin cleavage products, and lack of factor Va cofactor amplification (B. S. Schwartz, D. S. Fair, and T. S. Edgington, manuscript in preparation).

The synthesis by specifically induced monocytes of an activator of the coagulation pathway is consistent with fibrin deposition in inflammatory tissue lesions. Fibrin formation per se is responsible for the induration of delayed-type hypersensitivity reactions (13), in which monocytes are major effector cells. Human monocytes in the presence of lymphocytes respond to LPS or Ag/Ab by generating tissue factor or comparable procoagulant molecules (17, 18). In contrast, human monocytes respond to lymphocytes triggered by certain density classes of human plasma lipoproteins by expression of a prothrombinase (28, 36). The present description of an inducible murine monocyte prothrombinase provides a mechanism for direct generation of thrombin or a thrombinlike activity at inflammatory sites. This product clots fibrinogen; whether these prothrombin products are also capable of activating factors V (42) and VIII (43) and stimulating platelets (44), as well as fulfilling other functions of thrombin (45, 46) remains to be examined. Further insight to possible molecular mechanisms of interactions between components of the coagulation process and the immune system have been forthcoming from the studies of Geczy and Hopper (47) and Hopper et al. (48). Inhibition of guinea pig peritoneal macrophage migration correlated with lymphokine-dependent macrophage production of a tissue factorlike activity. These investigations have proposed that lymphokine-induced inhibition of peritoneal macrophage migration may be mediated by fibrin formation on the macrophage surface. The presence of a prothrombinase on the surface of murine monocytes provides a direct mechanism for such fibrin formation.

Summary

Murine lymphoid cells respond rapidly to bacterial lipopolysaccharide or antigen-antibody complexes to initiate or accelerate the blood coagulation pathways. The monocyte or macrophage has been identified as the cellular source, although lymphocyte collaboration is required for the rapid induction of the procoagulant response. This procoagulant activity is identified in the present study as a direct prothrombin activator, i.e., a prothrombinase. Studies with plasmas deficient in single coagulation factors demonstrate that the induced murine procoagulant activity effector molecule does not require factors XII, VIII, VII, X, or V, but does require prothrombin to transform fibrinogen to fibrin. This enzyme(s) produces limited proteolysis of prothrombin to yield thrombin or thrombinlike products that are functionally capable of converting fibrinogen to fibrin. The prothrombinase is undetectable in freshly isolated

murine peripheral blood mononuclear cells, but increases to maximal levels within 6 h after cells are exposed to bacterial lipopolysaccharide or soluble antigen/antibody complexes. Induced monocytes express the prothrombinase on their surface; however, the activity is increased fivefold by cellular disruption indicating a predominantly intracellular pool of enzyme. The prothrombinase is not secreted into the medium in an active form but is recovered with the cell membranes and small organelles. Cleavage of prothrombin by the monocyte prothrombinase requires the presence of calcium and is inhibited by 2.5 mM diisopropylfluorophosphate, suggesting that it may be a serine protease. Enzymatic activity is proportional to the concentration of monocytes, and the pH and thermal optima are in the physiologic range. This neutral protease exhibits first-order kinetics, and the cleavage of prothrombin increases linearly with time. Induction of activity appears most likely due to newly initiated synthesis of the enzyme per se rather than neutralization of an inhibitor or activation of a zymogen. Monocytes induced to express prothrombinase by this collaborative pathway are not induced to express plasminogen activator. We propose that this procoagulant effector molecule or monokine may be responsible for local fibrin deposition observed at sites of lipopolysaccharide- and antigen/antibody complex-induced inflammation and may contribute to the histogenesis and pathogenesis of these and certain other immunologically mediated tissue lesions.

References

1. North, R. J. 1978. The concept of the activated macrophage. *J. Immunol.* **121:**806.
2. Karnovsky, M. L., and J. K. Lazdins. 1978. Biochemical criteria for activated macrophages. *J. Immunol.* **121:**809.
3. Mantovani, B., M. Rabinovitch, and V. Nussenzweig. 1972. Phagocytosis of immune complexes by macrophages. Different role of the macrophage receptor sites for complement (C3) and for immunoglobulin (IgG). *J. Exp. Med.* **135:**780.
4. Simon, H. B., and J. H. Sheagren. 1971. Cellular immunity in vitro. I. Immunologically mediated enhancement of macrophage bactericidal capacity. *J. Exp. Med.* **133:**1377.
5. Rocklin, R. E., K. Bendtzen, and D. Greineder. 1980. Mediators of immunity: lymphokines and monokines. *Adv. Immunol.* **29:**55.
6. Gery, I., and B. H. Waksman. 1972. Potentiation of the T-lymphocyte response to mitogens. II. The cellular source of potentiating mediators. *J. Exp. Med.* **136:**143.
7. Chervenick, P. A., and A. F. Lobuglio. 1972. Human blood monocyte: stimulators of granulocyte and mononuclear colony formation *in vitro. Science (Wash. D. C.).* **178:**164.
8. Werb, Z., and S. Gordon. 1975. Secretion of a specific collagenase by stimulated macrophages. *J. Exp. Med.* **142:**346.
9. Werb, Z., and S. Gordon. 1975. Elastase secretion by stimulated macrophages. Characterization and regulation. *J. Exp. Med.* **142:**361.
10. Unkeless, J. C., S. Gordon, and E. Reich. 1974. Secretion of plasminogen activator by stimulated macrophages. *J. Exp. Med.* **139:**834.
11. Wyatt, H. V., H. R. Colten, and T. Borsos. 1972. Production of the second (C2) and fourth (C4) components of guinea pig complement by single peritoneal cells: evidence that one cell may produce both components. *J. Immunol.* **108:**1609.
12. Dvorak, H. F., M. C. Mihm, A. M. Dvorak, R. A. Johnson, E. J. Manseao, E. Morgan, and

R. B. Colvin. 1974. Morphology of delayed type hypersensitivity in man. I. Quantitative description of the inflammatory response. *Lab. Invest.* **31**:111.

13. Colvin, R. B., R. A. Johnson, M. L. Mihm, and H. F. Dvorak. 1973. Role of the clotting system in cell-mediated hypersensitivity. I. Fibrin deposition in delayed skin reactions in man. *J. Exp. Med.* **138**:686.

14. Levy, G. A., and T. S. Edgington. 1980. Lymphocyte cooperation is required for amplification of macrophage procoagulant activity. *J. Exp. Med.* **151**:1232.

15. Schwartz, B. S., and T. S. Edgington. 1981. Lymphocyte collaboration is required for induction of murine monocyte procoagulant activity by immune complexes. *J. Immunol.* **127**:438.

16. Levy, G. A., and T. S. Edgington. 1982. The major histocompatibility complex requirement for cellular collaboration in the murine lymphoid procoagulant response stimulated by bacterial lipopolysaccharide. *J. Immunol.* **128**:1284.

17. Lerner, R. B., R. Goldstein, and G. Cummings. 1971. Stimulation of human leukocyte thromboplastic activity by endotoxin. *Proc. Soc. Exp. Biol. Med.* **138**:145.

18. Rothberger, H., T. S. Zimmerman, H. L. Spiegelberg, and J. H. Vaughan. 1977. Leukocyte procoagulant activity. Enhancement of production in vitro by IgG and antigen-antibody complexes. *J. Clin. Invest.* **59**:549.

19. Rothberger, H., T. S. Zimmerman, and J. H. Vaughan. 1978. Increased production and expression of tissue thromboplastin-like procoagulant activity in vitro by allogenically stimulated human leukocytes. *J. Clin. Invest.* **62**:649.

20. Prydz, H., T. Lyberg, P. Detilex, and A. C. Allison. 1979. *In vitro* stimulation of tissue thromboplastin activity in human monocytes by immune complexes and lectins. *Thromb. Res.* **15**:465.

21. Muhlfelder, T. W., J. Niemetz, D. Kreutzer, D. Beebe, P. Ward, and S. I. Rosenfeld. 1979. C5 chemotactic fragment induces leukocyte production of tissue factor activity. A link between complement and coagulation. *J. Clin. Invest.* **63**:147.

22. Miletich, J. P., C. M. Jackson, and P. W. Majerus. 1978. Properties of the Factor Xa binding site on human platelets. *J. Biol. Chem.* **253**:6908.

23. Van Dieyen, G., G. Tans, J. Rosing, and H. C. Hemker. 1981. The role of phospholipid and Factor VIIIa in the activation of bovine factor X. *J. Biol. Chem.* **256**:3433.

24. Li, C. Y., K. W. Lan, and L. T. Yan. 1973. Esterases in human leukocytes. *J. Histochem. Cytochem.* **21**:1.

25. Netu, E., O. Westphal, O. Lundenitz, E. A. Gorzyski, and E. Eichenberger. 1956. Studies of enterobacterial lipopolysaccharides: effects of heat and chemicals on erythrocyte modifying, antigenic, toxic, and pyrogenic properties. *J. Immunol.* **76**:377.

26. Morrision, D. C., J. A. Louis, and W. O. Wiegle. 1976. Dissociation of anticomplementary and adjuvant properties of proteins derived from cobra venoms. *Immunology.* **30**:317.

27. Fair, D. S., E. F. Plow, and T. S. Edgington. 1979. Combined functional and immunochemical analysis of normal and abnormal human Factor X. *J. Clin. Invest.* **64**:884.

28. Schwartz, B. S., G. A. Levy, L. K. Curtiss, D. S. Fair, and T. S. Edgington. 1981. Plasma lipoprotein induction and suppression of the generation of cellular procoagulant activity in vitro. Two procoagulant activities are produced by peripheral blood mononuclear cells. *J. Clin. Invest.* **67**:1650.

29. Weber, K., and M. Osborn. 1969. The reliability of molecular weight determination by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* **244**:4406.

30. DiScipio, R. G., M. A. Hermodsen, and E. W. Davie. 1977. Activation of human factor X (Stuart Factor) by a protein from Russell's viper venom. *Biochemistry.* **16**:5253.

31. Doolittle, R. F., D. S. Schubert, and S. A. Schwartz. 1967. Amino acid sequence studies on artiodacryl fibrinopeptides. *Arch. Biochem. Biophys.* **118**:456.

32. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* **227:**680.

33. Levin, E. G., and D. J. Loskutoff. 1979. Comparative studies of the fibrinolytic activity of cultured vascular cells. *Thromb. Res.* **15:**869.

34. Jackson, C. M., and Y. Nemerson. 1980. Blood coagulation. *Annu. Rev. Biochem.* **49:**765.

35. Rickles, F. R., J. A. Hardin, F. A. Pitlick, L. W. Hoyer, and M. E. Conrad. 1973. Tissue factor activity in lymphocyte cultures from normal individuals and patients with hemophilia A. *J. Clin. Invest.* **52:**1427.

36. Levy, G. A., B. S. Schwartz, L. K. Curtiss, and T. S. Edgington. 1981. Plasma lipoprotein induction and suppression of the generation of cellular procoagulant activity in vitro. Requirements for cellular collaboration. *J. Clin. Invest.* **67:**1614.

37. Davie, E. W., K. Fujikawa, K. Kurachi, and W. Kisiel. 1979. The role of serine proteases in the blood coagulation cascade. *Adv. Enzymol.* **49:**277.

38. Nelsestuen, G. L. 1976. Role of γ-carboxyglutamic acid: an unusual protein transition required for the calcium dependent binding of prothrombin to phospholipid. *J. Biol. Chem.* **251:**5648.

39. Werb, Z., M. J. Banda, and P. A. Jones. 1980. Degradation of connective tissue matrices by macrophages. I. Proteolysis of elastin, glycoproteins, and collagen by proteases isolated from macrophages. *J. Exp. Med.* **152:**1340.

40. Greineder, K. K., K. J. Connorton, and J. R. David. 1979. Plasminogen activator production by human monocytes. I. Enhancement by activated lymphocytes and lymphocyte products. *J. Immunol.* **123:**2808.

41. Osterud, B., V. Lindahl, and R. Seljelid. 1980. Macrophages produce blood coagulation factors. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **120:**41.

42. Kane, W. H., and P. W. Majerus. 1981. Purification and characterization of human coagulation factor V. *J. Biol. Chem.* **256:**1002.

43. Vehar, G. A., and E. W. Davie. 1980. Preparation and properties of bovine factor VIII (antihemophilic factor). *Biochemistry.* **19:**401.

44. Marguerie, G. A., E. F. Plow, and T. S. Edgington. 1979. Human platelets possess an inducible and saturable receptor specific for fibrinogen. *J. Biol. Chem.* **254:**5357.

45. Kisiel, W. 1979. Human plasma protein C. Isolation, characterization, and mechanisms of activation by α-thrombin. *J. Clin. Invest.* **64:**761.

46. Schwartz, M. L., S. V. Pizzo, R. L. Hill, and P. A. McKee. 1971. The subunit structures of human plasma and platelet Factor XIII (Fibrin stabilizing factor). *J. Biol. Chem.* **246:**5851.

47. Geczy, G. L., and K. E. Hopper. 1981. A mechanism of migration inhibition in delayed type hypersensitivity reactions. II. Lymphokines promote procoagulant activity of macrophages *in vitro. J. Immunol.* **126:**1059.

48. Hopper, K. E., C. L. Geczy, and W. A. Davies. 1981. A mechanisms of migration inhibition in delayed type hypersensitivity reactions. I. Fibrin deposition on the surface of elicited peritoneal macrophages *in vivo. J. Immunol.* **126:**1052.