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

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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 docu-
mented (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 charac-
teristics 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 depo-
sition. 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 concentra-
tion. 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

* Supported by research grants CA-28166 and HL-16411 from the National Institutes of Health.
‡ Supported by training grant HL-07195 from the National Institutes of Health.
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1 Abbreviations used in this paper: Ag/Ab, antigen/antibody complexes; DFP, diisopropylfluorophosphate; 
LPS, lipopolysaccharide (Escherichia coli 0111:B4, butanol extracted); PAGE, polyacrylamide gel electro-
phoresis; PBM, peripheral blood mononuclear cells; PCA, procoagulant activity; SDS, sodium dodecyl 
sulfate; TBS, Tris-buffered saline (0.01 M Tris, 0.15 M NaCl, pH 7.4).
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% CO2-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 gyratory 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% CO2 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 NaN3 (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 asp 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% NaN₃ 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 CaCl₂ 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 x 90 cm) equilibrated in PBS (0.01 M phosphate, pH 6.0, 0.14 M NaCl), 1 mM benzamidine, 0.02% NaN₃. 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 recalculated 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 CaCl₂, 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 10⁶ 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 ¹²⁵I-prothrombin and 0.01 ml of 25 mM CaCl₂ 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 ¹²⁵I. 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 ¹²⁵I-prothrombin (0.01 ml 100 nM) and CaCl₂ (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 ¹²⁵I-Prothrombin Cleavage by Stimulated Monocytes. For kinetic studies 0.05 ml of cellular homogenates (2 x 10⁶ Ag/Ab or LPS-stimulated PBM/ml tris-buffered saline [TBS]) were incubated at 37°C with 0.01 ml ¹²⁵I-prothrombin (100 nM) (0.045 potential U thrombin), 0.01 ml hirudin (0.2 U) (Sigma Chemical Co.), 0.02 ml CaCl₂ (25 mM). At selected intervals, the reaction was stopped by the addition of EDTA and SDS, and ¹²⁵I-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 x 10⁶ 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 CaCl₂ (0.01 ml in buffer of same pH), and 125I-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 125I-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 × 10⁶ LPS- or Ag/Ab-triggered PBM were combined with 0–4 × 10⁶ 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 125I-prothrombin-cleaving activity.

Plasminogen Activator. Homogenates of 1–6 × 10⁶ PBM in 0.5 ml TBS were analyzed for plasminogen activator by the 125I-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/10⁶ cells). This was equivalent whether the cells were dispersed over a 2-cm² surface area of a plastic culture plate or incubated in a small settled pellet of ~0.1 cm² 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

| 10⁶ PBM incubated with:* | Intact viable cells | Cell homogenate |
|-------------------------|---------------------|----------------|
|                         | Clotting time $^\dagger$ | PCA ($^\ddagger$) | Clotting time $^\dagger$ | PCA ($^\ddagger$) |
|                         | s mU/10⁶ cells | s mU/10⁶ 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              |

* 10⁶ PBM incubated with indicated stimulus at 37°C 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.
INDUCIBLE MONOCYTE PROTHROMBINASE

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

| Cell stimulus | NHP† | Procoagulant activity: (mU) plasma deficient in:§ |
|---------------|------|--------------------------------------------------|
|               |      | XII | VIII | VII | X | V | II | +II||
| LPS           | 31   | 30  | 30   | 30  | 31 | 30 | >120 | 31 |
|               | (1450) | (1500) | (1520) | (1560) | (1480) | (1520) | (<4) | (1500) |
| Ag/Ab         | 30   | 30  | 30   | 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.

[1 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 ¹²⁵I-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 ¹²⁵I-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 ¹²⁵I-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 ¹²⁵I-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, $\beta$-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 (-----); CaCl$_2$ and TBS (-----); CaCl$_2$ and 150 ng/ml (final concentration) purified human factor Xa (-----).

Table III

| 10$^6$ PBM incubated with* | Fibrinogen 4 mg/ml | Prothrombin 100 $\mu$g/ml | CaCl$_2$ 25 mM | Clotting time |
|-----------------------------|--------------------|--------------------------|-----------------|--------------|
| 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.
Inhibition of inducible prothrombinase of murine monocytes by DFP. Homogenates of $1 \times 10^8$ 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. 2.

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 $\mu$g/ml soluble Ag/Ab (●) or 10 $\mu$g/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.

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), 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.
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 $5 \times 10^6$ 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 $4 \times 10^6$ 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/microorganelle 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 | Normal human plasma‡ | Purified components§ | Percent $^{125}$I-prothrombin cleaved/10$^6$ cells ||
| | s/mlU | s | 51 | 3 | 98 |
| 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°C 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.
homogenate from $1 \times 10^3$ 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 CaCl$_2$. 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 $1 \times 10^6$ PBM possessing prothrombinase activity* | Percent $^{125}$I-prothrombin cleavage‡ | Percent augmentation (+) or inhibition (−) of $^{125}$I-prothrombin cleavage |
|---|---|---|
| 0 | 46 | — |
| $1 \times 10^6$ | 43 | −6.5 |
| $2 \times 10^6$ | 50 | +8.7 |
| $3 \times 10^6$ | 43 | −6.5 |
| $4 \times 10^6$ | 48 | +4.4 |

* $1 \times 10^6$ 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 ($1 \times 10^6$) of LPS- or Ag/Ab-induced PBM were combined with $0-4 \times 10^6$ 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 × 10⁶ 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 ¹²⁵I-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 procoagulant 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. ¹²⁵I-prothrombin, which had been incubated with unstimulated PBM, remained intact by SDS-PAGE. However, the addition of PCA-positive PBM to ¹²⁵I-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.

We wish to thank Barbara Borders for expert secretarial assistance.

Received for publication 21 December 1981.

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