

MONOCYTES CAN BE INDUCED BY
LIPOPOLYSACCHARIDE-TRIGGERED T LYMPHOCYTES TO
EXPRESS FUNCTIONAL FACTOR VII/VIIa PROTEASE
ACTIVITY

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The monocyte has been perceived as a relatively inactive member of the monocyte/macrophage series, i.e., a cell in transport to an anatomic site where differentiation to the macrophage will occur. Modification of this perception is merited in light of the ready capacity of monocytes, as well as their tissue-localized progeny, the elicited macrophage, to initiate and propagate coagulation protease pathways (reviewed in references 1–4). The human monocyte given appropriate direct collaboration by bacterial lipopolysaccharide (LPS),¹ immune complex-stimulated T lymphocytes (5–7), or a lymphokine (8, 9) from allogeneically or antigen-stimulated T cells (10, 11), produces and expresses on its surface a cell membrane lipoprotein, i.e., tissue factor, the initiating cofactor of the extrinsic coagulation protease pathway (12, 13). This initiating event is attributable to the calcium-dependent binding of Factor VII or VIIa to tissue factor (14, 15) to form a molecular complex capable of efficiently converting the zymogen Factor X to its active form by virtue of the limited proteolytic specificity of Factor VII and VIIa. This protease, the only coagulation protease that is proven to be active in the zymogen form (16), also can proteolytically activate Factor IX, of the intrinsic pathway (17).

In the human (18) and the mouse (19) an independent procoagulant protease, monocyte prothrombinase, has been identified. This protease can directly mediate the conversion of prothrombin to thrombin. Other studies have implicated additional cellular proteases including a rabbit tumor cell-associated Factor X-activating cysteine protease (20), a Factor X-activating protease in LPS-stimulated rabbit Kupffer cells (21), a vitamin K-dependent protease activity associated with Lewis lung carcinoma (22), and a murine macrophage protease with Factor X-activating properties (23). We have implicated the synthesis of valid

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¹ *Abbreviations used in this paper:* EDTA, ethylene diaminetetracetic acid; Mo, monocytes; LPS, bacterial lipopolysaccharide; PBM, peripheral blood mononuclear cells; PCA, procoagulant activity; T_{ind}, inducer T cells.

Factor VII/VIIa by human peripheral blood mononuclear cells (PBM) following LPS stimulation (24).

In the present study we demonstrate that appropriately induced human monocytes can produce and assemble Factor VII or VIIa in a proteolytically active form on their surface in the absence of an exogenous source of this trace plasma protein. This is the first documentation of such a response with direct evidence that the protein responsible for proteolytic activation of Factor X is indeed Factor VII/VIIa. Evidence also is provided that this effector function can be induced in monocytes by signals from appropriately stimulated T cells. The generation of factor VII or VIIa (VII/VIIa) represents a further expansion of the known proteolytic repertoire of cells of the monocyte/macrophage series and has implications for the activation of the coagulation protease pathways in inflammatory lesions by lymphoid cells.

Materials and Methods

Cell Isolation and Culture. Platelet-poor PBM were prepared from healthy donors from heparinized (5 U/ml) venous blood by centrifugation at 100 g for 20 min. After removal of the platelet-rich plasma, the cells were washed once with incomplete medium (Leibovitz's L-15 medium; Irvine Scientific, Santa Ana, CA) or RPMI-1640 (Gibco Laboratories, Grand Island, NY) prepared in pyrogen-free water containing 2 mM glutamine (Gibco) and 50 μ g/ml gentamycin (Schering Corp., Kenilworth, NJ) and recentrifuged at 100 g. All culture media and reagents were devoid of LPS contamination (<1 ng/ml) by the limulus amoebocyte lysate assay (E-toxate, Sigma Chemical Co., St. Louis, MO).

PBM were isolated from either whole blood or platelet-poor blood cells by centrifugation on Ficoll-Hypaque (Sigma Chemical Co., and Winthrop Laboratories, Menlo Park, CA, respectively) prepared in pyrogen-free water at a density of 1.074 g/ml) at 425 g for 25 min at 17°C. The cells at the interphase were collected, washed twice with incomplete medium, and resuspended at 1×10^6 cells/ml in either serum-free medium or complete medium (the latter containing 10% heat-inactivated fetal calf serum). PBM contained <1 platelet per mononuclear cell, <1% polymorphonuclear leukocytes, and >99% excluded trypan blue.

Isolated monocytes and Leu-3a⁺ lymphocytes were kindly isolated in our laboratory by Dr. Heikki Helin (7) and were prepared as follows: monocytes were isolated from PBM by receptor-mediated adherence to plasma fibronectin on gelatin-coated surfaces (7, 25). 1-ml aliquots containing 10^6 PBM in complete medium were incubated for 40 min at 37°C in 16-mm tissue culture wells (Costar, Cambridge, MA) coated with fibronectin by incubation with autologous plasma after coating the wells with gelatin. Nonadherent cells were removed 1 h later, and the adherent cells were washed twice with warm serum-free medium. The adherent cells were >97% positive for nonspecific esterase (26) and represented $62.9 \pm 1.7\%$ of the original esterase-positive cells. Nonadherent cells were subjected to another cycle of adherence (overnight at 37°C) to deplete them of residual monocytes. After the second cycle of adherence, the nonadherent lymphocytes were incubated with a murine monoclonal antibody to Leu-3a, a cell surface epitope specific for a helper/inducer subset of human T cells (27). Using fluorescein isothiocyanate-labeled F(ab)₂ fragments of goat anti-mouse Ig, the Leu-3a positive cells were collected after sorting on a FACS II or FACS IV (Becton-Dickinson, Mountain View, CA) as previously described (7).

PBM at 1×10^6 /ml, were incubated in 12 \times 75 mm polypropylene tubes at 37°C in either complete medium for 18 h or serum-free medium for 5 h. In experiments to assess the potential role of culture medium in the responses, RPMI-1640 was replaced with L-15. Results were qualitatively comparable. Monocytes ($\sim 1 \times 10^5$) purified from 1×10^6 PBM were cultured in polystyrene 16-mm plastic Costar plates, either alone or with 8.5×10^5 Leu-3a⁺ T cells in 1 ml of complete medium for 4 h at 37°C. Bacterial LPS

(*Escherichia coli* 0111:B4, butanol-extracted) was added at final (optimal) concentrations of 10 $\mu\text{g/ml}$ to cultures in complete medium and at 10 ng/ml to cultures containing incomplete medium.

Coagulation Assays. The basal procoagulant activity (PCA) of freshly isolated (zero time) PBM was determined by immediately centrifuging 1×10^6 cells in 1 ml at 2,000 g for 15 min, discarding the supernatant and freezing the cell pellets at -70°C for assay of the total PCA. Cultured cells were handled in an identical manner at the termination of the incubation period. For total PCA assay the cell pellets were lysed in a final volume of 0.33 ml by one of two methods: either repeated freezing and thawing followed by sonication as described previously (28), or by detergent lysis. The latter involved incubating the cell pellets at 37°C in 0.1 ml of 150 mM NaCl and 25 mM Hepes (Hepes saline) containing 15 mM octyl glucoside (Calbiochem-Behring Corp., La Jolla, CA). After 10 min, the volume was increased to 0.33 ml by the addition of Hepes saline and the samples were placed on ice. There was no effect of the detergent on assays of any of the coagulation proteases at the final concentration in the assays; and maximal PCA was observed for incubation periods in octyl glucoside of 8–20 min at 37°C . For assay of viable cell PCA after culture of the PBM at 1×10^6 cells/ml, the cells were centrifuged at 2,000 g for 10 min in polypropylene tubes, resuspended in Hepes saline and the contents of two tubes pooled and adjusted to a final concentration of 1×10^7 cells per milliliter in Hepes saline.

PCA was quantitated by a one-stage clotting assay. Cell lysates or viable cells (0.05 ml) or thromboplastin standard (0.05 ml) was added to a 12×75 mm borosilicate tube that contained 0.05 ml of 20 mM CaCl_2 , and warmed to 37°C . After the addition of 0.05 ml of citrated platelet-poor normal plasma (diluted twofold in 20 mM citrate, 150 mM NaCl, pH 7.4), the time required for fibrin clot formation was visually recorded. In selected experiments, normal human plasma was replaced with human plasmas genetically deficient in specific coagulation proteases (George King Biological Co., Overland Park, KS) to establish the entry point of the monocyte PCA into the coagulation protease sequence. Clotting times were converted to units of PCA from log-log plots of serial dilutions of a tissue factor standard (rabbit brain thromboplastin; Difco Laboratories, Detroit, MI), which were assayed as previously described and for which 1,000 mU/ml samples gave 50 ± 2 s (28).

Purified Coagulation Factors. Factor X and prothrombin were isolated from Cohn Fraction III (a gift from Dr. W. Brockway, Cutter Laboratories) as previously described (29). Human fibrinogen was kindly provided by Dr. E. F. Plow (Research Institute of Scripps Clinic). Human Factor VII was prepared by the method of Broze and Majerus (30). Bovine Factor V was the generous gift of Drs. K. G. Mann and M. E. Nesheim (31).

Chromogenic Assay for Factor X Activation. Formation of activated Factor X by rabbit brain thromboplastin or stimulated cell lysates was determined in a two-stage assay using the Factor Xa chromogenic substrate S-2222 (BZ-Ile-Glu-Gly-Arg-pNA) (Kabi Diagnostica, Stockholm, Sweden). In the first stage of Factor X activation 0.2 ml of citrated normal or specific factor-deficient plasma (diluted 1:10 with 10 mM Tris, 150 mM NaCl, pH 7.4), 0.1 ml of tissue factor standard or cell lysate, and 0.008 ml of 0.5 M CaCl_2 were incubated at 37°C . After 3 min, 160 mU of hirudin (Sigma Chemical Co.) in 0.6 ml of 50 mM Tris, 227 mM NaCl, 10 mM EDTA, pH 8.3, was added to inactivate the thrombin generated in the first stage. After the addition of 0.1 ml of 2.7 mM S-2222, the initial rate of release of *p*-nitroaniline was monitored at 405 nm at 37°C . *p*-Nitroaniline release was linear for at least 5 min.

Immunoglobulin Fractions. Rabbit antibody to human Factor VII was prepared by repeated immunization with highly purified Factor VII in complete, then incomplete adjuvant (32). An IgG fraction of this antibody was isolated by ammonium sulfate precipitation (45% saturation) and chromatography on DEAE-cellulose (DE-52, Whatman) equilibrated with 15 mM phosphate (pH 8.0). The specificity of this antibody has been extensively characterized and confirmed (32). Normal rabbit serum IgG was prepared in an identical manner. Goat antibody specific for human Factor VII was generously provided by Dr. S. Paul Bajaj (University of California, San Diego).

Results

Freshly isolated cells expressed basal levels of total cellular PCA that were quite low, e.g. 14 ± 3 mU/ 10^6 cells. However, incubation of PBM for 5 h with LPS induced a 40-fold increase in total cellular PCA (567 ± 200 mU/ 10^6 cells) over initial basal content or 30-fold relative to the unstimulated cultured PBM (19 ± 3 mU/ 10^6 cells). Comparable increases were observed for intact, viable cells. No significant differences in PCA generated were observed for cells incubated 5 h in serum-free medium as compared to 18 h in complete medium; and there was no significant recovery of PCA in the cell-free ($100,000 g$ for 1 h) culture supernatants. The cellular PCA appeared to be predominantly of intracellular origin, since viable cells expressed only 5–20% of the total functional activity, consistent with previous observations (6, 28). For assay of total PCA cell pellets were either disrupted by freeze-thaw and sonicated, or lysed in 15 mM octyl glucoside for 10 min at 37°C . Detergent lysates contained 100–200% of the PCA of that obtained by freeze-thaw and sonication. Therefore detergent lysis was used predominantly for these studies and key observations were confirmed by freeze-thaw and sonication. Molecules responsible for PCA were not solubilized by the octyl glucoside, because activity present in the detergent lysate of LPS-stimulated PBM was distributed only in the residual cellular structures recovered by centrifugation at low force and vesicles recovered by centrifugation at $100,000 g$ for 1 h. Octyl glucoside solutions added to plasma to a final concentration of 20 mM, a 12-fold higher final concentration than in the adopted method, had no effect on function of the coagulation assays.

To characterize the molecular species of induced PCA, cell lysates of LPS-stimulated PBM were assayed for acceleration of coagulation of various factor-deficient human plasmas in one-stage coagulation assays. As shown in Table I, plasmas deficient in Factors VIII or IX supported coagulation initiated by: (a) a

TABLE I
Coagulation Factor Dependence of LPS-Induced PCA in Human PBM

	Tissue factor*	LPS-stimulated PBM [‡]	Control PBM plus Xa [§]
	s	s	s
Normal	44 ± 6	46 ± 4	36 ± 1
Factor-deficient			
VII	47 ± 5	40 ± 7	42 ± 1
IX	54 ± 2	55 ± 4	51 ± 2
VII	166 ± 14	76 ± 6	54 ± 5
X	>200	>200	40 ± 1
II	>200	>200	>200

* Rabbit brain thromboplastin was used as a source of tissue factor and was diluted to achieve a PCA that was comparable to the PCA of LPS-stimulated PBM. Data of four separate experiments.

[‡] 1×10^6 PBM were cultured with 10 ng/ml LPS for 5 h in serum-free medium (three experiments) or with 10 $\mu\text{g}/\text{ml}$ LPS in complete medium for 18 h (six experiments).

[§] 1×10^6 control cultured PBM plus 0.56 ng/ml of purified Factor Xa (two experiments).

^{||} One-stage clotting time is expressed as the mean seconds \pm the standard deviation.

tissue factor source; (b) LPS-stimulated PBM, or (c) control PBM plus Factor Xa, as well as did control normal pooled plasma, indicating no requirement for these proteins in the protease pathway. In contrast, LPS-stimulated cells did not accelerate clotting of Factor X- or II-deficient plasmas and this was similar for the tissue factor source. Competence of the protease pathway was restored in Factor X-deficient plasma by repletion with purified Factor X, and in Factor II-deficient plasma by repletion with purified Factor II (data not shown). Tissue factor only slightly accelerated the coagulation of Factor VII-deficient plasma, i.e., from 220 s to 166 s (Table I). However, the LPS-stimulated PBM lysates significantly accelerated coagulation of recalcified Factor VII-deficient plasma, i.e., from >200 s to 76 s, suggesting that these cells contained either legitimate Factor VII/VIIa or a protease with Factor VIIa-like activity. The cellular PCA was partially dependent on Factor VII because the rate of the response, 76 s, was less than that for normal control plasma, 46 s. These stimulated PBM did not initiate coagulation of Factor X-deficient plasma even though PBM with added Xa directly induced coagulation. Thus the initiating activity in the absence of Factor VII was dependent on the presence of Factor X and was a Factor X-activating activity. The activities are synthesized *de novo* since all PCA activity was abolished by both actinomycin D and by cycloheximide (5).

To obviate the possibility that the observed Factor VII-independent PCA was attributable to carryover of plasma Factor VII during cell isolation and to provide evidence that Factor VII is not produced constitutively by monocytes, the ability of these control cells to bind exogenous Factor VII and to accelerate the coagulation of Factor VII-deficient plasma in the presence of a tissue factor source was quantitated. Iodinated Factor VII was added to and incubated with blood and the cell population before Ficoll-Hypaque separation. The isolated PBM cells were associated with <0.06% of the total label added, which was equivalent to <1 pg/10⁶ monocytes, a quantity too low to account for the observed Factor VII-like activity. As illustrated in Fig. 1, the coagulation of Factor VII-deficient plasma by tissue factor was not accelerated by the addition of control cell lysates. This indicated that control cultured PBM did not possess

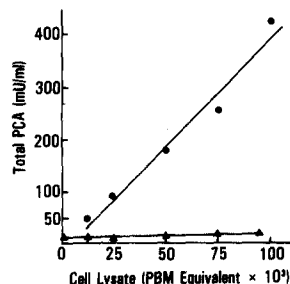


FIGURE 1. LPS-stimulated human peripheral blood mononuclear cells express Factor VII-like procoagulant activity in a dose-dependent manner. PBM cells were cultivated for 5 h in serum-free medium in the presence (●) or absence (Δ) of 10 ng/ml LPS and disrupted by octyl glucoside. Increasing concentrations of cellular lysates were tested for PCA activity in Factor VII-deficient plasma. The LPS-stimulated cells (which express tissue factor) were assayed directly. The control unstimulated cells were assayed in the presence of 1,000 mU/ml of tissue factor. The absence of effect in the latter is taken as evidence that there was not cellular carryover of Factor VII and that it was not produced by unstimulated cells.

Factor VII, nor could they provide some otherwise inapparent substitute for the Factor VII requirement. In contrast, LPS-stimulated PBM alone expressed a Factor VII-independent PCA in a dose-dependent manner as exhibited by the linear PCA response with increasing cell lysate. Similar results have been observed for all of the above experiments with intact viable cells, and cultivation of the cells in the presence or absence of heat-inactivated fetal calf serum in either of two different media did not have a significant effect.

Enzymatic Characterization. The partial Factor VII independence of the cellular PCA was quantitatively assayed in a two-stage amidolytic assay using Factor X as substrate for the cellular PCA and the chromogenic substrate S-2222 to assay Factor Xa generation. LPS-stimulated PBM added to Factor VII-deficient plasma generated Factor Xa at a rate of 40% of that observed for normal human plasma substrate, whereas tissue factor was unable to generate Factor Xa activity ($\leq 10\%$) using either Factor VII- or X-deficient plasmas as substrate (Table II). The addition of purified Factor VII to Factor VII-deficient plasma quantitatively reconstituted the full capacity of the induced PCA to generate Factor Xa. The activity of LPS-stimulated PBM was minimally expressed ($< 10\%$) in Factor X-deficient plasma, and could be fully reconstituted by the addition of purified Factor X. In contrast to tissue factor, LPS-stimulated PBM also were able to generate Factor Xa using purified Factor X in the absence of other proteins (Table II). The generation of this Factor Xa activity was 35% of the maximal activity of that observed for an intact extrinsic pathway in the presence of normal plasma. Thus, this represented the Factor X-activating protease alone without the contribution of cellular tissue factor; and indeed the rate of Factor Xa formation was not significantly different from that observed for Factor VII-deficient plasma. It should be noted that these data were highly reproducible whether the monocyte PCA response was elicited in vitro in the absence or presence of heat-inactivated fetal calf serum and were not influenced by the type of culture medium used.

TABLE II
Activation of Factor X by LPS-stimulated PBM*

Plasma	Tissue factor [‡]		LPS-stimulated PBM [§]	
	A ₄₀₅ /min	Percent	A ₄₀₅ /min	Percent
Normal	0.054	100	0.060	100
VII-def	0.005	9	0.024	40
VII-def + VII [¶]	ND [†]		0.056	93
X-def	0.006	10	0.005	9
X-def + X ^{**}	ND		0.060	100
Purified factor X ^{**}	0.002	4	0.021	35

* Factor Xa generation was measured using the chromogenic substrate S2222, which is specific for Factor Xa as described in Materials and Methods.

[‡] Rabbit brain thromboplastin diluted to give a clotting time of 48 s in normal human plasma.

[§] 1×10^6 PBM incubated for 5 h at 37°C with 10 ng/ml of LPS in serum-free medium. The cell lysate had a clotting time of 47 s in normal plasma.

[¶] Factor VII at 0.5 $\mu\text{g/ml}$.

[†] Not determined.

** Factor X at 6.5 $\mu\text{g/ml}$.

** Factor X at 6.5 $\mu\text{g/ml}$ assayed in the absence of plasma and cell extract.

TABLE III
*Factors X and V Are Required by LPS-stimulated PBM for Fibrin Formation in a System of Purified Components**

Clotting factors				Clotting time	
Fibrinogen (800 µg/ml)	II (32 µg/ml)	Va [‡] (3.8 µg/ml)	X (2.6 µg/ml)	Control PBM + Xa [§]	LPS-stimulated PBM [¶]
				s	s
+	+	0	0	>240	>240
+	+	+	0	19	>240
+	+	+	+	ND [†]	31
+	+	0	+	ND	111

* Reaction mixtures (0.125 ml) were incubated at 37°C and contained 7.5 mM CaCl₂.

[‡] 100 µg/ml of Factor V was activated by thrombin, and the thrombin removed by ion exchange chromatography.

[§] 1 × 10⁶ PBM were incubated for 5 h at 37°C in serum-free medium. The cell pellet was solubilized in detergent and Factor Xa was added to 0.56 ng/ml. Clotting time in normal plasma, 36 s.

[¶] 1 × 10⁶ PBM were incubated with 10 ng/ml of LPS for 5 h at 37°C in serum-free medium. Clotting time in normal plasma, 38 s.

[†] Not determined.

The ability of the induced cells to directly activate Factor X was demonstrated using a purified component assay that contained fibrinogen, prothrombin, Factor Va, and Factor X (Table III). Control cell lysates failed to activate Factor X, as control PBM contained only 2% of the PCA expressed by LPS-stimulated cells (data not shown). LPS-stimulated PBM expressed minimal PCA (>240 s) in the presence of fibrinogen, prothrombin, Factor Va, and Ca²⁺. With the addition of Factor X, a profound acceleration (31 s) was observed with LPS-stimulated PBM, and indicated that conversion of Factor X to Factor Xa had occurred. In the presence of Factor Va, acceleration of the coagulation rate was observed for the control cell lysates plus purified Factor Xa. This indicated that Factor Va was absent from the control cells, but was required for maximal rates of prothrombin generation in this assay system. Also, in the absence of added Factor Va the activity of LPS-stimulated PBM exhibited a very limited capacity to accelerate the coagulation response, i.e., a time of 111 s, indicating a requirement for Factor Va.

Calcium Requirements. The Factor X-activating PCA present in LPS-stimulated PBM cell lysates was entirely dependent upon available calcium ions. This was demonstrated in a two-stage assay. In the first stage, LPS-stimulated PBM were incubated with Factor X in the absence or presence of Ca²⁺ for 5 min at 37°C. In the second stage, the other purified components and additional CaCl₂ were added, and the time recorded for the appearance of fibrin. The results are given in Table IV. With either 1.7 mM or 3.4 mM Ca²⁺ present for Factor X activation during the first stage, the PCA from LPS-stimulated PBM was comparable to that observed in normal human plasma. <10% was observed with LPS-stimulated PBM, when Ca²⁺ was omitted for Factor X activation during the first stage. This minimal expression of PCA (<10%) could result from the activation of Factor X during the second stage of the assay that contained 7.4 mM CaCl₂.

It has been demonstrated that both human Factor VII and VIIa bind equiva-

TABLE IV
Calcium Requirement for Direct Factor X Activation by LPS-stimulated PBM*

Ca ²⁺ (mM)	Factor Va (0.74 μg/ml)	Clotting time	Total PCA
		<i>s</i>	<i>mU/10⁶ PBM</i>
0	-	252 ± 17	<0.5
0	+	94 ± 3	24 ± 3
1.7	+	52 ± 2	280 ± 46
3.4	+	50 ± 1	320 ± 20

* In the first stage, LPS-stimulated PBM were incubated for 5 min at 37°C with Factor X at 8.6 μg/ml in the absence or presence of the indicated concentrations of CaCl₂ to allow for Factor X activation. After the addition of 32 μg/ml prothrombin, 0.74 μg/ml Factor Va, 800 μg/ml fibrinogen and additional CaCl₂, the clotting time was recorded. The final concentration of CaCl₂ in the second stage clotting assay was from 7.4 to 8.8 mM.

lently to LPS-stimulated monocytes, that this binding is Ca²⁺ dependent (15), and that it presumably represents binding to tissue factor (14). To determine whether the association of the Factor X-activating protease with monocytes involved divalent ions, PBM (1 × 10⁶/ml) were cultured for 5 h in serum-free media in the absence or presence of LPS. After culture the intact viable cells were washed, incubated for 10 min at 37°C in 1.0 ml of Hepes saline containing 0–5 mM EDTA, and washed twice in Hepes saline before assay of the viable cell PCA. Whereas the EDTA wash had no effect on the viable cell PCA of control PBM, at 3.0–5.0 mM EDTA the LPS-stimulated PCA retained on intact cells was reduced by >80% (data not shown). The divalent ions involved appeared to be tightly bound, because 1–2 mM EDTA only slightly diminished the cell-bound PCA. The cell-associated Factor X-activating activity of LPS-stimulated PBM was dissociable in a manner similar to Factor VII.

Immunochemical Specificity. To determine whether the Factor X-activating molecule was indeed Factor VII or VIIa, LPS-stimulated PBM were incubated with rabbit IgG containing neutralizing antibodies specific for human Factor VII (32). It was first necessary to verify that the Factor VII-specific Ig could neutralize Factor VII bound to LPS-stimulated PBM. After 5 h of culture at 37°C in serum-free media in the absence or presence of 10 ng/ml LPS, viable PBM at 1–2 × 10⁶ cells/ml were resuspended in 0.2 ml of Hepes saline containing 3 mM CaCl₂ and 50 ng/ml of purified human Factor VII, and incubated for 20 min at 4°C. After removal of non-cell associated Factor VII and treatment with normal or immune IgG, the PCA expressed by intact cells was quantitated. LPS-stimulated intact cells that were incubated with purified Factor VII accelerated the coagulation of both normal and Factor VII-deficient plasmas, when compared with control cells (Table V). PCA in Factor VII-deficient plasma was 25–30% of that measured with normal plasma. This activity could be neutralized by anti-Factor VII IgG and not normal IgG. This was confirmed by a second anti-Factor VII IgG (kindly provided by Dr. Paul Bajaj).

With this evidence for functional neutralization in hand, we examined the ability to neutralize the PCA of LPS-stimulated PBM that were devoid of

TABLE V
Neutralization of PCA on LPS-stimulated PBM by Factor VII Specific Antibody

Tissue*	Factor VII [‡]	Ig [§]	Viable cell PCA (mU/10 ⁶ PBM)	
			NHP	Factor VII-deficient plasma
Control PBM	+	Normal	2.1	<1
Control PBM	+	Anti-VII	2.0	<1
LPS-PBM	+	Normal	50.0	13
LPS-PBM	+	Anti-VII	3.5	<1
LPS-PBM	0	Normal	27.0	9.9
LPS-PBM	0	Anti-VII	3.0	<1

* PBM at 1×10^6 /ml were cultured for 5 h at 37°C in serum-free medium in the absence or presence of 10 ng/ml of LPS. After incubation 2×10^6 PBM were collected by centrifugation and the cell pellets resuspended in 0.2 ml of HEPES saline containing 3 mM CaCl₂.

[‡] Factor VII was added to the cell pellets at 50 ng/ml and the cells incubated for 20 min at 4°C, washed twice, and resuspended in 0.2 ml of HEPES saline containing 3 mM CaCl₂.

[§] 75 µg of rabbit anti Factor VII IgG or normal rabbit IgG was added to the cells. The cells were incubated for 30 min at 37°C, washed twice, resuspended in HEPES saline, and assayed for viable cell PCA in normal and Factor VII-deficient human plasma in one-stage clotting assays.

NHP, normal human plasma.

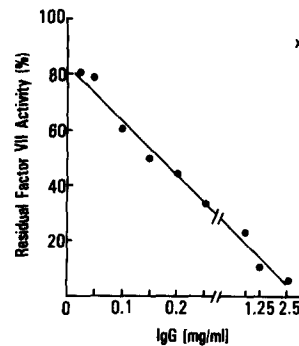


FIGURE 2. Neutralization of cellular Factor VII/VIIa-like protease activity in a dose-dependent fashion by IgG antibodies to human Factor VII/VIIa. Peripheral blood mononuclear cells (2×10^6) were induced in serum-free culture with 10 ng/ml LPS, incubated for 30 min at 37°C with varying concentrations of IgG antibodies to Factor VII (●) or 2.5 mg/ml normal IgG (X), and analyzed for residual Factor VII/VIIa-like PCA in Factor VII-deficient plasma.

exogenous purified Factor VII. This was neutralized also by the Factor VII antibody (Table V). When this was examined in a quantitative titration of antibody, a dose-dependent neutralization of cellular Factor VII/VIIa-like protease activity was observed (Fig. 2). This was true for two independent sources of antibodies to highly purified Factor VII. Thus, the Factor VII/VIIa-like PCA

of LPS-stimulated PBM was immunochemically identified as human Factor VII or its activated derivative, Factor VIIa.

T Cell Requirement. Previous studies in our laboratory have established that the cellular source of the LPS-induced tissue factor-like PCA expressed by human PBM is the monocyte. However, the expression of this monocyte PCA after exposure to LPS has appeared to be dependent upon the presence of T lymphocytes (5, 7) when the monocytes are isolated without activation. Because no information was available regarding the cellular pathways for induction of monocyte Factor VII/VIIa, the T cell requirement has been characterized. Peripheral blood monocytes were purified by adherence to plasma fibronectin on gelatin-coated plastic surfaces to a relative purity of >97%, as indicated by nonspecific esterase staining. A T lymphocyte inducer/helper population was isolated from nonadherent lymphocytes by fluorescence-activated cell sorting with monoclonal antibody Leu-3a followed by fluoresceinated F(ab)₂ fragments of goat anti-mouse Ig. Reconstituted cells (Leu-3a⁺ T cells plus monocytes at an 8:1 ratio) responded with a 20-fold increase in PCA relative to monocytes alone (270 vs. 13 mU/10⁶ PBM) after LPS stimulation. Therefore, a Leu-3a⁺ T cell population was required for the LPS induction of this monocyte PCA. Similarly to LPS-stimulated whole PBM, the PCA of the T cell-monocyte mixture in one-stage clotting assays was partially dependent on Factor VII and entirely dependent on Factor X.

When assayed in normal human plasma in the two-stage amidolytic assay, the LPS-stimulated reconstituted cultures of purified T Leu-3a⁺ lymphocytes and monocytes generated five times more Factor Xa-dependent activity than did monocytes alone (Table VI). In the absence of T cells nearly all of the amidolytic activity (91%) expressed by LPS-exposed monocytes was independent of Factor VII. In the presence of LPS-stimulated Leu-3a⁺ T cells, monocytes expressed

TABLE VI
*Activation of Factor X by Monocytes Requires T Cell Collaboration**

Plasma	LPS-T _{ind} -Mo [‡]		LPS-Mo [§]	
	A ₄₀₅ /min	Percent	A ₄₀₅ /min	Percent
Normal	0.049	100	0.011	100
VII-def	0.019	39	0.010	91
VII-def + VII [†]	0.048	98	ND [†]	—
X-def	0.010	20	0.005	45
X-def + X ^{**}	0.047	96	ND	—
X ^{‡‡}	0.002	4	ND	—

* Factor Xa generation was measured using the chromogenic substrate S-2222 as described in Materials and Methods.

‡ Monocytes (Mo) purified by adherence from 1 × 10⁶ PBM were reconstituted with 8.5 × 10⁵ Leu-3a⁺ helper/inducer T cells (T_{ind}) and cultured for 4 h at 37°C in complete medium in the presence of 10 μg/ml LPS. Clotting time in normal plasma, 57 s.

§ Monocytes, purified by adherence from 1 × 10⁶ PBM, were cultured for 4 h at 37°C in complete medium in the presence of 10 μg/ml LPS. Clotting time in normal plasma, 122 s.

† Factor VII at 0.5 μg/ml.

‡ Not determined.

** Factor X at 6.5 μg/ml.

‡‡ Factor X at 6.5 μg/ml assayed in the absence of plasma.

39% of the activity of normal human plasma when assayed in Factor VII-deficient plasma, and 20% of the activity when assayed in Factor X-deficient plasma. These activities were fully restored to the level observed in normal plasma by repletion with each of the purified coagulation proteins. The amount of the Factor VII-independent PCA expressed by the reconstituted cells (39%, Table VI) was similar to that of LPS-stimulated PBM (40%, Table II). In addition, the PCA of the reconstituted cells could be neutralized by antibodies to Factor VII in a dose-dependent fashion (data not shown). Therefore, LPS-stimulated monocytes in the presence of Leu-3a⁺ T inducer/helper lymphocytes expressed a Factor VII-independent PCA that was comparable in all respects to the PCA of LPS-stimulated PBM.

Discussion

One of the major pathways of the immune response involves T cell-instructed responses by cells of the monocyte/macrophage series. One of the effector limbs of this pathway culminates in thrombin formation and local fibrin deposition in immunologic lesions as demonstrated by Dvorak, Colvin, and colleagues (33–36) for delayed cutaneous hypersensitivity. Implication of this pathway has been further substantiated by the attenuating effects of anticoagulation (37). Neutral proteases are synthesized by monocytes and macrophages under the influence of T cells and their products (38, 39). These properties are well within the currently recognized repertoire of responses of these effector cells and appear central not only to their role in immunologically mediated inflammation but as well to the initiation of intravascular coagulation (40, 41).

Monocytes, the early migratory cells of this lineage, are noteworthy for vigorous T cell-dependent responses to a variety of biologically relevant stimuli, including responses that initiate the coagulation protease network (1–4, 24, 37, 38, 42, 43). Whereas many of the proteolytic responses of the monocyte/macrophage are direct, namely secretion of an active protease, the recruitment of the coagulation protease network has until recently been thought to involve only the synthesis and cell surface expression of tissue factor, a nonenzymatic cofactor that binds Factor VII and VIIa, and enhances their functional activity (14–16, 44). Recently, two additional roles of the monocyte have been described. First, monocytes can assemble the second macromolecular complex in the extrinsic pathway, the prothrombinase complex, which is composed of Factor Xa and Va and which functions to proteolytically activate prothrombin (45). Indeed, monocytes contain significant quantities of the requisite cofactor, i.e., Factor V (45). This series of molecular assemblies is essential for propagation of the extrinsic coagulation pathway. Second, when properly instructed by T cells, monocytes can synthesize and express a functional prothrombinase that is a membrane-associated serine protease unrelated to other known coagulation proteases (18, 19). This latter protease represents primary initiation rather than propagation, but is distinct from the molecular biology of the response characterized here.

In the present study we provide substantial evidence that participation of the monocyte in the extrinsic coagulation pathway is more complex than originally suspected. It not only synthesizes tissue factor but also can produce Factor VII/

VIIa and assemble it on the cell surface in a functional form. Whether this is single chain zymogen VII or two chain-activated VIIa is not known but is currently under analysis. However, both forms are active (16) when bound to tissue factor via calcium ions (14, 44). We assume that the Factor VII/VIIa may be associated with tissue factor on the cell surface, though this will require specific analysis.

Others have described cellular Factor X-activating activity in a variety of neoplastic and non-neoplastic cells (20-23). Such proteolytic activity could represent Factor VII/VIIa; however, Gordon and Gross (20) have demonstrated that the activity from rabbit lung carcinoma cells was a cysteine protease, thus not Factor VII/VIIa. The evidence from studies of rabbit (21) and murine (23) macrophages suggests the possibility of Factor VIIa-like activity, but these studies did not establish whether the responsible molecules were indeed Factor VII/VIIa. Other observations, such as those with human allogeneic lymphocyte (42) or IgG-induced (43) responses, suggested the possibility of procoagulant activity in addition to tissue factor; however, the identity of such additional activity was not delineated.

Support for the production and expression of Factor VII/VIIa by LPS-induced T cell-instructed monocytes was derived from several lines of evidence. Enzymatic activity of the cell-associated procoagulant activity directly cleaved Factor X as demonstrated in both coagulation and amidolytic assays. The proteolytic response was calcium dependent, as would be expected for a γ -carboxylated protease of the coagulation pathway, and it was abolished by monospecific antibodies to Factor VII/VIIa. In addition, the Factor VII/VIIa was bound to the cell surface by a tight calcium adduct, since relatively high concentrations of EDTA were necessary to dissociate the protease from the intact viable procoagulant active monocyte. Synthesis of Factor VII/VIIa was not constitutive since there was no functional activity in unstimulated intact or disrupted cells, even when an exogenous source of tissue factor was provided. As for the induction of tissue factor (7), T cells of the Leu-3a positive helper/inducer subclass have been implicated in the collaborative induction of Factor VII/VIIa in monocytes when properly isolated in a basal state. Whether the induction of Factor VII/VIIa is mediated by precisely the same signals from the T cell that induce tissue factor is unknown. Indeed, until the communicative requirements are delineated at the molecular level it will be difficult to determine whether the linkage of the induction of tissue factor and Factor VII/VIIa is a function that is intrinsic to the monocyte/macrophage, or elective and at the discretion of the T cell.

The present observations further expand on the hypothesis that cells of the monocyte/macrophage lineage are intimately involved in initiation of the coagulation protease network. The functional necessity for the cellular synthesis of the proteins of the extrinsic cascade might seem less than obvious in view of the availability of these zymogens in plasma and body fluids. However, the low concentrations of many of these may preclude effective assembly of the extrinsic pathway in extravascular sites. However, cellular synthesis could provide a greater efficiency of assembly into proteolytically functional complexes and would contribute the advantage of coordinate synthesis and cell surface expres-

sion of multiple molecules of the extrinsic pathway. The preservation of such complex pathways of signals for induction suggests that they may be significant.

Summary

In the present study we demonstrate that human monocytes can be induced by the model stimulus, lipopolysaccharide (LPS), to produce and assemble on their surface functional Factor VII/VIIa. This protease was not induced in relatively purified monocytes alone following exposure to LPS; but was induced in the presence of Leu-3a positive helper/inducer T cells. The Factor VII/VIIa protease activity represented 35–40% of the potential initiating activity for the extrinsic coagulation pathway and was demonstrated using functional coagulation assays, as well as in amidolytic assays for the activation of Factor X. This activity of cell-bound Factor VII/VIIa appeared to involve a tight adduct of calcium. The identity of the Factor X-activating protease as Factor VII/VIIa was confirmed by the capacity of antibody specific for Factor VII/VIIa to neutralize the cell-bound protease. Further propagation of the extrinsic pathway following generation of Factor Xa required addition of exogenous Factor Va.

These results expand the repertoire of proteases that have been identified with appropriately triggered cells of the monocyte/macrophage series, and suggest that initiation and propagation of the extrinsic coagulation protease network on induced monocytes involves not only expression of the initiating cofactor molecule, tissue factor, but also production of Factor VII and its organization into the molecular assembly. Thus, in the absence of exogenous Factor VII/VIIa a directly proteolytic effector cell can be generated. Further molecular assembly of the extrinsic pathway on the monocyte surface sequentially expands the proteolytic capacity of this response. The synthesis and assembly of the extrinsic activation complex by the monocyte and its derived progeny, the macrophage, provides a mechanism by which coagulation is initiated under T cell instruction at sites of immunologic responses.

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