

# The mandatory role of complement in the endotoxin-induced synthesis of tissue thromboplastin in blood monocytes

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Fresh isolated blood cells recombined with normal heparinized plasma and then incubated with endotoxin, induced a 100-fold increase in monocyte tissue thromboplastin synthesis. In contrast, recombination of these cells with heat inactivated plasma, cobra venom factor-treated plasma,  $\text{Ca}^{2+}$ -free plasma, or BioRex 70-treated plasma (plasma free of Clq and D) before incubation with endotoxin, failed to induce monocyte synthesis of tissue thromboplastin. These results strongly support the hypothesis that complement is required for endotoxin stimulation of blood monocyte synthesis of tissue thromboplastin.

*Monocyte*

*Thromboplastin synthesis*

*Complement*

*Endotoxin*

## 1. INTRODUCTION

Bacterial lipopolysaccharides (endotoxins) induce tissue thromboplastin synthesis in monocytes [1–3]. We have found that 75% of the newly synthesized thromboplastin becomes available on the surface of the monocytes [4], and thereby may cause a rapid and highly potent activation of the coagulation system leading to severe disseminated intravascular coagulation in septicemia [5].

In addition to endotoxins, a variety of components such as lectins, immune-complexes, allogeneic cells, C5a and C3b, have all been stated to induce a procoagulant activity (probably tissue thromboplastin) in isolated monocytes [6–10].

However, experiments with monocytes in cell cultures incubated with endotoxin in the presence and absence of anti-C5 antibodies, showed no inhibitory effect of the antibody [9]. These investigators excluded therefore the complement components C5–C9 as a mediator of the endotoxin on monocytes.

This investigation concludes that the endotoxin effect is dependent on the complement system. In contrast to previous reports our results are obtained by using unmodified monocytes; i.e., newly drawn blood monocytes recombined with plasma subjected to various treatments.

## 2. MATERIALS AND METHODS

### 2.1. Blood collection

Human blood was collected into heparin (5 U/ml); 0.025 M EGTA + 0.025 M  $\text{MgCl}_2$  pH 7.4; 0.2% EDTA (pH 7.4); 0.01 M sodium citrate–citric acid (pH 7.4) [10], in polycarbonate tubes. These are all final concentrations of the anticoagulants.

### 2.2. Isolation and thromboplastin quantitation of mononuclear cells

Mononuclear cells were isolated on Lymphopaque (a gift from Nyegaard, Oslo) as in [1]. The cells were washed once by resuspending the band containing the mononuclear cells in 0.15 M NaCl and collected by centrifugation at  $1500 \times g$  for 10 min. These cell preparations contained ~30% monocytes and 70% lymphocytes and some platelets. The cell sediment for 1 ml blood was resuspended in 0.15 M NaCl, and the amount of tissue thromboplastin in the monocytes after freezing and thawing was determined by incubating the test sample with purified factor VII and factor X as in [12]. The factor Xa activity generated in these incubation mixtures was measured with a clotting assay. This activity was then related to the factor Xa activity obtained in the same system by testing

dilutions of a crude tissue thromboplastin preparation from human brain [4]. The undiluted thromboplastin was defined as 100% activity. We have shown that only monocytes possess thromboplastin activity [4]. Furthermore, the contaminating lymphocytes and platelets had no effect on our assay system for thromboplastin activity [4].

### 2.3. Endotoxin stimulation of monocytes

Whole blood or blood cells (1 ml) recombined with plasma pretreated in various ways, in 1.3 cm  $\times$  8.0 cm polycarbonate tubes was incubated with *Escherichia coli* 0.26:B6 endotoxin (obtained from Difco Laboratories, Detroit MI) for 2 h (or various time-intervals) at 37°C. The mononuclear cells were then immediately isolated. A stock solution of 4 mg endotoxin/ml was prepared by dissolving it in 0.15 M NaCl and kept at 4°C. In some experiments (indicated in section 3) endotoxins from Sigma (St Louis MO) were used.

### 2.4. Inactivation of complement factors in the alternative and classical pathways

Heparinized blood was centrifuged at 3500  $\times$  g for 10 min at room-temperature. The plasma was pipetted off into plastic tubes. One part of the plasma was heated in the waterbath at 50°C for 20 min to inactivate the B-component of the alternative pathway. The other part of the plasma sample was incubated at 56°C for 30 min to inactivate both the alternative and classical complement systems.

### 2.5 Removal of plasma complement factor by BioRex chromatography

To 5 ml heparinized plasma in polycarbonate tubes were added 0.5 g filtered BioRex 70 (Bio-Rad Labs., Richmond CA) that had been equilibrated with buffer composed to 0.082 M NaCl, 2 mM EDTA and 0.05 M sodium phosphate (pH 7.3) [15]. After stirring for 30 min the BioRex 70 was removed from the plasma by centrifugation at 12000  $\times$  g for 10 min.

### 2.6. Cobra venom factor

Anticomplementary factor from cobra venom was obtained from Cordis Labs. (Miami FL). In most of our experiments the venom from *Naja naja kaouthia* (cat.no. 750-007) was used. The

venom from *Naja haje* (cat.no. 750-005) was also tested, but this cobra venom factor had less effect than the venom from *Naja naja kaouthia*.

## 3. RESULTS

### 3.1. Requirement of $Ca^{2+}$ in endotoxin-induced synthesis of tissue thromboplastin in monocytes in blood

Table 1 shows that EDTA and EGTA as anti-coagulants block the effect of endotoxin on monocytes in whole blood. As shown in [4], endotoxin is capable of inducing thromboplastin synthesis in monocytes of citrate blood. However, compared with thromboplastin activity in monocytes of heparinized blood incubated with endotoxin (see fig. 1), a substantial inhibition of the endotoxin effect is also caused by citrate anti-coagulant.

Table 1

The tissue thromboplastin activity of monocytes isolated from blood collected into various anticoagulants, and incubated with endotoxin

Endotoxin ( $\mu$ g/ml)	Tissue thromboplastin activity/ $10^3$		
	EDTA	EGTA + MgCl <sub>2</sub>	Citrate
0.050	0.14	0.05	1.9
0.5	0.30	0.05	6.6
5.0	0.07	0.07	12.0
50.0	0.09	0.04	20.0

Blood samples (1 ml) in 0.2% Na<sub>2</sub> EDTA (pH 7.4) or 0.025 M EGTA + 0.025 M MgCl<sub>2</sub> (pH 7.4) or 0.01 M sodium citrate-citric acid (pH 7.4) (final conc.) were incubated with various concentrations of endotoxin for 2 h at 37°C. The mononuclear cells were isolated and their thromboplastin activity measured

Fig. 1 illustrates the effect of incubating 1 ng endotoxin/ml heparin blood of 4 normal donors. A remarkable variation among individual responses to endotoxin is seen. The blood donor with the highest response to endotoxin was routinely observed even to generate some tissue thromboplastin in the monocytes of heparinized blood-samples incubated in the absence of endotoxin. Blood from the same donors were also incubated

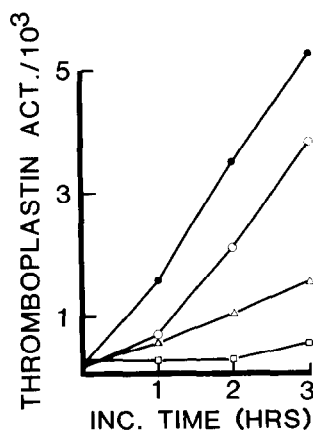


Fig. 1. The synthesis of tissue thromboplastin in monocytes of heparinized blood from 4 donors incubated with 1 ng endotoxin/ml at 37°C. At the time-intervals shown, 1 ml blood samples were pipetted off and the mononuclear cells isolated. The monocyte numbers (17) in the test samples were not significantly different.

with PHA and other endotoxins (*E. coli* 0111:34, *E. coli* 0127:B8, *Salmonella enteridis*, *Salmonella typhosa*, and *Serratia marcescens*) and the same responses were seen with these stimulating agents.

### 3.2. Failure of endotoxin to induce synthesis of tissue thromboplastin in monocytes in heat-inactivated plasma

Blood cells were separated and recombined with either fresh untreated plasma, or plasma either incubated at 50°C for 20 min or 56°C for 30 min. The recombined samples were then subjected to incubation with endotoxin for 2 h. The monocytes of the incubated samples were isolated and the thromboplastin content quantitated. The average results of 4 expt were as follows. When plasma had been incubated at 56°C, 50°C or 20°C the resultant activities of thromboplastin were, respectively,  $1.4 \pm 1.9/10^3$ ,  $10.0 \pm 2.7/10^3$  and  $17.1 \pm 4.2/10^3$  per  $10^6$  monocytes, as expressed in percentage of our crude human standard tissue thromboplastin. Thus heat inactivation at 56°C for 30 min of the plasma abolished the endotoxin effect on the monocytes. The heat treatment is known to destroy the complement activity in plasma. Heat inactivation of plasma at 50°C for 20 min, a procedure known to destroy the activity of the alternative pathway in the complement system, partially re-

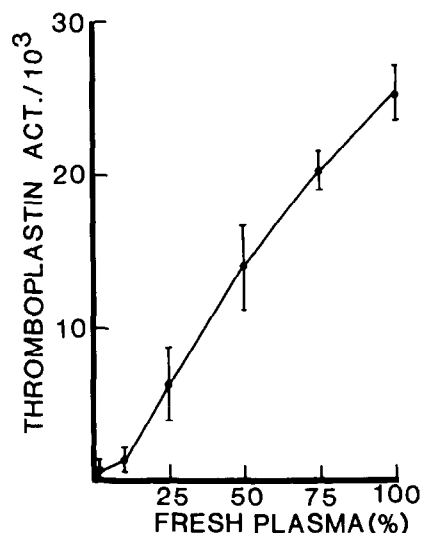


Fig. 2. The requirement of fresh plasma for the induction of tissue thromboplastin synthesis in endotoxin-stimulated monocytes. Washed blood cells were recombined with heat-inactivated plasma (56°C for 30 min) mixed with increasing ratio of fresh plasma, and incubated for 2 h with 0.5 µg endotoxin/ml at 37°C. Then the mononuclear cells were isolated and their thromboplastin content measured. Monocytes counts were performed on each sample, but no change in the recovery of monocyte of the different blood samples was observed.

moved the stimulating effect of endotoxin in blood.

The necessity for fresh plasma in order to obtain a stimulating effect of endotoxin on the synthesis of tissue thromboplastin is shown in fig. 2. An almost linear relationship was found between the amount of tissue thromboplastin and the amount of fresh plasma present in the incubation mixture of the blood cells and endotoxin.

### 3.3. The effect of endotoxin on blood cells in plasma depleted of complement factors by cobra venom factor or adsorption with BioRex 70-resin

Plasma was incubated with various concentrations of cobra venom factor (CVF) for 3 h at 37°C. Such plasma was then combined with washed blood cells and incubated with 0.5 µg endotoxin/ml for 2 h. After the incubation, the mononuclear cells were isolated and their content of tissue thromboplastin determined. The following results were found:  $40 \pm 5.3/10^3$ ,  $8.1 \pm 3.1/10^3$ ,

$5.1 \pm 2.3/10^3$  and  $3.1 \pm 1.5/10^3$  thromboplastin activity per  $10^6$  monocytes, as expressed in percentage of our crude human standard tissue thromboplastin (av. 3 expt). These values correspond to the value found in blood cells recombined, either with untreated plasma or plasma preincubated with 1.0, 2.0 and 4.0 U cobra venom factor/ml, respectively.

After removal of factor D and C1q from plasma by chromatography on BioRex 70 [13], the endotoxin failed to induce the synthesis of tissue thromboplastin when washed blood cells were recombined with this plasma (table 2). A small activation seems to occur following washing the cells as seen from lines 2 and 4 of table 2. This activation may account for most of the activity in the test sample shown in line 5.

Table 2

The effect of endotoxin on blood cells recombined with normal plasma and subjected to chromatography on Bio-Rex 70

Test samples	Tissue thromboplastin activity/ $10^3$
Blood + endotoxin	34.8
Blood + 0.15 M NaCl	0.3
Blood-cells + plasma	
+ endotoxin	32.1
Blood-cells + plasma	
+ 0.15 M NaCl	2.5
Blood-cells + Bio-Rex 70-absorbed plasma	
+ endotoxin	3.9
Blood cells + Bio-Rex 70-absorbed plasma	
+ 0.15 M NaCl	1.4

Blood (1 ml) or combined samples of 0.5 ml washed blood-cells + 0.5 ml fresh plasma or Bio-Rex 70-absorbed plasma were incubated with (0.5  $\mu$ g/ml) or without endotoxin for 2 h at 37°C. The mononuclear cells were isolated and their tissue thromboplastin content measured

#### 4. DISCUSSION

These results show for the first time evidence for a mandatory role of complement factors in the endotoxin-induced synthesis of tissue throm-

boplastin in monocytes in whole blood. The finding that monocytes, in the absence of plasma factors, or in the presence of 56°C heat-inactivated plasma, CWF-treated plasma, or BioRex 70-absorbed plasma, failed to produce tissue thromboplastin when incubated with endotoxin, strongly suggest to us a requirement for complement factors in this reaction. The need for  $Ca^{2+}$  also supports this hypothesis. The low activity generated in citrated blood incubated with endotoxin as compared to heparinized blood, probably indicates that there is sufficient free  $Ca^{2+}$  on the surface of the cells.

No effect of anti-C5 antibody was shown in monocyte-plasma system incubated with endotoxin [9]. However, the anti-C5 antibodies do not inhibit the initial steps in the complement cascade. Furthermore, the initial formation of antibody-antigen complexes may also activate the complement system before full neutralization of C5 is obtained.

A direct effect of endotoxin on monocytes has been shown in [1-3,14-16]. However, these experiments have all been carried out in cell cultures where the monocytes probably already have changed their properties. Cultured monocytes are known to produce complement factors, which could account for the endotoxin effect in the absence of an external complement source. Conceivably, cultured monocytes might be induced to synthesize tissue thromboplastin in the total absence of complement, in contrast to freshly drawn blood monocytes.

Our experiments indicate that the complement system is important for inducing activation of the coagulation system by its interaction with the monocytes. This event, resulting in exposed thromboplastin on the surface of the monocytes, is probably the only cause of the fulminant reactions seen in some Gram-negative septicaemia patients. Furthermore, preliminary data have revealed that thrombin through its activation of complement is capable of inducing tissue thromboplastin synthesis in monocytes. This mechanism for thromboplastin generation is now being explored.

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