

# Role of NF $\kappa$ B in the Mortality of Sepsis

Hubert Böhler,\* Feng Qiu,\* Thomas Zimmermann,† Youming Zhang,\* Thomas Jilmer,‡ Daniela Männel,§ Bernd W. Böttiger,\* David M. Stern,|| Rüdiger Waldherr,\* Hans-Detlev Saeger,‡ Reinhard Ziegler,\* Angelika Bierhaus,\* Eike Martin,\* and Peter P. Nawroth\*

\*Department of Anesthesiology, Department of Medicine, and Department of Pathology, University of Heidelberg, D-69120 Heidelberg, Germany; †Department of Internal Medicine and Department of Visceral-Thoracic-Vascular Surgery, University of Dresden, D-01307 Dresden, Germany; ‡Institute of Pathology/Tumorimmunology, University of Regensburg, D-93053 Regensburg, Germany; and ||Department of Physiology, Columbia University, New York 10032

## Abstract

Binding activity for nuclear factor kappa B (NF $\kappa$ B) consensus probes was studied in nuclear extracts from peripheral blood mononuclear cells of 15 septic patients (10 surviving and 5 not surviving). Nonsurvivors could be distinguished from survivors by an increase in NF $\kappa$ B binding activity during the observation period ( $P < 0.001$ ). The increase in NF $\kappa$ B binding activity was comparable to the APACHE-II score as a predictor of outcome. Intravenous somatic gene transfer with an expression plasmid coding for I $\kappa$ B $\alpha$  was used to investigate the role of members of the NF $\kappa$ B family in a mouse model of endotoxemia. In this model, increased NF $\kappa$ B binding activity was present after injection of LPS. Intravenous somatic gene transfer with I $\kappa$ B $\alpha$  given before LPS attenuated renal NF $\kappa$ B binding activity and increased survival. Endothelial cells and monocytes/macrophages were the major target cells for somatic gene transfer, transfected with an average transfection efficiency of 20–35%. Tissue factor, a gene under regulatory control of NF $\kappa$ B, was induced by LPS. Somatic gene transfer with a reporter plasmid containing the functional tissue factor promoter demonstrated NF $\kappa$ B-dependent stimulation by LPS. Intravenous somatic gene transfer with I $\kappa$ B $\alpha$  reduced LPS-induced renal tissue factor expression, activation of the plasmatic coagulation system (decrease of thrombin–antithrombin III complexes) and renal fibrin/fibrinogen deposition. Somatic gene transfer with an expression plasmid with tissue factor cDNA in the antisense direction (in contrast to sense or vector alone) also increased survival. Furthermore, antisense tissue factor decreased renal tissue factor expression and the activation of the plasmatic coagulation system. (*J. Clin. Invest.* 1997. 100:972–985.) Key words: sepsis • NF $\kappa$ B • tissue factor • coagulation

## Introduction

LPS released by gram-negative bacteria is the most frequent cause of septic shock, affecting ~ 400,000 patients in the United States annually (1). The host response to LPS and in-

tact gram-negative organisms has been shown to involve multiple immune/inflammatory effector mechanisms, including expression of cytokines (2), arachidonic acid metabolites (3), and reactive oxygen and nitrogen intermediates (4). At the level of transcriptional regulation, LPS is known to activate complexes of proteins which bind to nuclear factor kappa B (NF $\kappa$ B)<sup>1</sup>-regulatory DNA sequences. Members of the NF $\kappa$ B family, induced by cytokines or directly by reactive oxygen intermediates, are believed to play a central role in LPS-mediated lethality (2, 5–9). However, direct proof of the role of members of the NF $\kappa$ B family in vivo is missing. Two particular gene products with a central role in the septic response are TNF $\alpha$  and tissue factor (10–15).

TNF $\alpha$  is a product of mononuclear cells exposed to LPS and its receptors are present on a broad range of cells, resulting in widespread effects. The pathophysiologic relevance of such TNF $\alpha$ -related mechanisms to tissue injury is supported by observations that inhibition of TNF $\alpha$  processing to the active form, passive immunization with neutralizing anti-TNF $\alpha$  antibodies, or gene deletion of the 55-kD TNF $\alpha$  receptor increase survival in animal models of endotoxemia (16–23). However, clinical studies using TNF $\alpha$ -neutralizing antibodies in the treatment of sepsis failed to show clear physiological or survival benefits in the overall study population (24–26).

Tissue factor, the principal initiator of coagulation in vivo, is closely tied to the host response in sepsis. Consumptive coagulopathy, resulting from tissue factor-mediated activation of the procoagulant pathway, is a consequence of LPS–cellular interactions and a cause of disseminated intravascular coagulation with resultant thrombotic and hemorrhagic complications (27–29). In vitro studies have shown the tissue factor gene to be under control of a *cis*-acting NF $\kappa$ B site (12–15, 30–34) and TNF $\alpha$  directly contributes to activation of this transcription factor leading to upregulation of tissue factor expression (12–15, 35–44).

That tissue factor is important in determining the outcome of sepsis is evident from the protective effect of inhibitory anti-tissue factor and anti-Factor VII/VIIa antibodies in *Escherichia coli* models of sepsis (45–48). The existence of a relationship between TNF $\alpha$  and tissue factor expression in vivo is supported by experiments showing that neutralization of TNF $\alpha$  reduces activation of coagulation in vivo (49). However, it remains unclear whether tissue factor expression in vivo depends on NF $\kappa$ B-mediated *de novo* transcription or whether the increased permeability of endothelial cells in sepsis leads to the exposure of cryptic tissue factor (50). If *de novo* transcription is required for the induction of tissue factor, then the

Address correspondence to H. Böhler, M.D., Dept. of Anesthesiology, Im Neuenheimer Feld 110, D-69120 Heidelberg, Germany. Phone: 49-6221-566351; FAX: 49-6221-565345.

Received for publication 29 July 1996 and accepted in revised form 20 June 1997.

*J. Clin. Invest.*

© The American Society for Clinical Investigation, Inc.  
0021-9738/97/09/0972/14 \$2.00

Volume 100, Number 5, September 1997, 972–985

<http://www.jci.org>

1. *Abbreviations used in this paper:* EMSA, electrophoretic mobility shift assay; Luc, luciferase; NF $\kappa$ B, nuclear factor kappa B; TAT, thrombin–antithrombin III complexes.

members of the NFκB family are likely candidates responsible for this effect.

This study demonstrates that nuclear extracts from PBMC harvested from nonsurviving septic patients show increased nuclear binding activity of NFκB to NFκB consensus oligonucleotide probes compared with survivors. In an endotoxemia model of LPS-treated mice, suppression of NFκB activation by intravenous somatic gene transfer with an IκB-overexpressing plasmid attenuated the LPS-mediated induction of tissue factor and increased survival. These data indicate a direct role for protein complexes activating expression of NFκB-regulated genes in the acute response to sepsis and endotoxemia.

## Methods

### Patients

A description of the patients, including age, sex, underlying disease, and the daily APACHE-II scores, is given in Table I. The patients presented were treated in the Department of Visceral-Thoracic-Vascular Surgery in Dresden, Germany (T.Z. and H.D.S.). The study was approved by the ethics committee of the University of Dresden, Germany, according to the guidelines of the Helsinki declaration.

### Animal model

Female BALB/c mice, aged 10–12 wk, 18–20 g (Charles River Wiga, Sulzfeld, Germany) were injected intraperitoneally with a mixture of *E. coli* LPS (0111:B4; Sigma Chemie, Deisenhofen, Germany; 1.75 μg in 0.1 ml sterile PBS, pH 7.4) and D-galactosamine (Sigma Chemie; 15 mg in 0.1 ml sterile PBS), in order to sensitize them to the lethal effects of LPS (51, 52). Mortality was monitored after 4, 8, 12, 16, 20, and 24 h. No further mortality was seen after 24 h.

### Plasmids

The pXT1 vector (Stratagene, Heidelberg, Germany) was used for constructing the tissue factor expression system. Murine tissue factor cDNA was generously provided by Dr. Nathans (Johns Hopkins University, Baltimore, MD; 53). The full-length mouse tissue factor cDNA was inserted into the XbaI site of the pXT1 vector from 3' to

5' for the tissue factor antisense construct (pXTF-as) or from 5' to 3' for the tissue factor sense construct (pXTF-s) (44) (Fig. 1). The pGLTF<sub>4</sub>-luciferase (LUC) expression plasmid, driven by the tissue factor promoter fragment (bp -278 to bp +121, pL4) (54) was constructed by inserting the fragment into the multiple-cloning site of pGL<sub>2</sub>-Luc (Promega, Heidelberg, Germany), from which the SV<sub>40</sub> promoter was removed (Fig. 1). The promoterless Luc-control vector pGL<sub>2</sub>-basic (Promega) served as control. The IκBα (pRC/CMVMAD-3wt) expression plasmid (55) was provided by Dr. P.A. Baeuerle (Tularik Inc., San Francisco, CA). The mutated jun plasmid pDB7, derived from the point mutant Mut14 (56), was generously provided by Dr. D. Bohmann (EMBL, Heidelberg, Germany). The β-galactosidase control plasmid pSV-β-Gal was obtained from Promega.

### Preparation of human PBMC

Human PBMC were separated after venipuncture from 20 ml whole blood, anticoagulated with 3.8% sodium citrate (9:1, vol/vol), by centrifugation on Ficoll Paque® (Pharmacia, Freiburg, Germany) according to the manufacturer's instructions. PBMC fractions were analyzed microscopically and independently counted by two investigators (A.B. and T.I.). Before electrophoretic mobility shift assays (EMSA), the amount of PBMC was adjusted to 2 × 10<sup>6</sup> PBMC/ml.

### Preparation of murine PBMC

Murine PBMC were separated using a similar method as for human PBMC isolation. For each time point, 5 ml whole blood was collected and pooled from 10 mice, anticoagulated with 3.8% sodium citrate (9:1, vol/vol), loaded carefully onto 5 ml of a Ficoll Paque® Plus gradient (Pharmacia) and centrifuged at 500 g without brakes at room temperature for 30 min according to the manufacturer's instructions. The mononuclear band was aspirated, washed with PBS, and analyzed microscopically by two investigators (F.Q. and A.B.). 10<sup>6</sup> PBMC were seeded onto gelatin-coated glass chamber slides (Nunc, Naperville, IL), and incubated overnight in RPMI medium containing 10% FCS, before immunohistochemistry was performed.

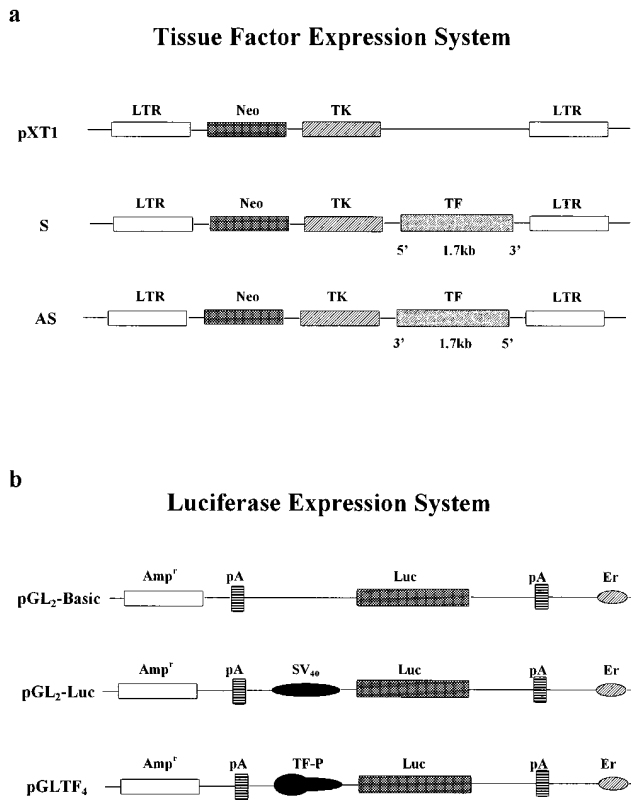
### EMSA

For EMSA, nuclear proteins were harvested by the method of Andrews (57). 2 × 10<sup>6</sup> isolated PBMC were lysed in 400 μl cold buffer A (10 mM Hepes-KOH, pH 7.9, at 4°C, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl,

Table I. Description of Patients

Patient No.	Age	Sex	Cause of sepsis	APACHE-II score								
				D1*	D2	D3	D4	D5	D6	D8	D10	D14
Survivors												
1	26 yr	M	Multiple trauma	8	6	6	9	10	11	13	12	12
2	38 yr	M	AHNP <sup>‡</sup>	2	9	2	2	3	2	0	0	0
3	56 yr	M	Chronic pancreatitis	10	11	11	9	8	8	8	8	
4	64 yr	F	Cholangitis	13	10	8	10	7	7	4	4	6
5	58 yr	M	Chronic pancreatitis	16	14	19	13	12	14	13	15	9
6	65 yr	M	Sternotomy	25	23	20	20	20	14	14	18	15
7	36 yr	M	Rupture of the spleen	20	21	14	16	14	5	5	5	5
8	40 yr	M	AHNP	19	3	2	2	2	3	3	2	2
9	41 yr	M	Ulcerative colitis	15	10	4	3	2	5	6	9	5
10	21 yr	F	Multiple trauma	4	0	0	5	7	9	7	14	12
Nonsurvivors												
11	23 yr	F	Multiple trauma	29	27	28	28	28	31	32		
12	64 yr	M	Peritonitis, lung carcinoma	12	15	17	17	21	21	20	28	
13	60 yr	M	Rupture of the spleen	15	16	16						
14	48 yr	M	Duodenal insufficiency	13	15	15	16	12	13	10	12	7
15	59 yr	M	Kidney and lung failure	29	28	33	28	25	27	29	21	23

\*D1, day 1, etc. <sup>‡</sup>AHNP, acute hemorrhagic necrotizing pancreatitis.



**Figure 1.** Scheme of expression plasmids. (a) Tissue factor expression system. *pXTI*, vector plasmid; *S*, sense tissue factor expression plasmid *pXTF-s*; *AS*, antisense tissue factor expression plasmid *pXTF-as*; *LTR*, Moloney murine leukemia virus long terminal repeats; *Neo*, neomycin resistance; *TK*, herpes simplex thymidine kinase promoter; *TF*, mouse tissue factor. (b) Luc expression system. *SV<sub>40</sub>*, *SV<sub>40</sub>* promoter; *Er*, *SV<sub>40</sub>* enhancer; *TF-P*, tissue factor promoter; *Amp<sup>r</sup>*,  $\beta$ -lactamase; *pA*, synthetic poly(A) signal.

0.5 mM DTT, 0.2 mM PMSF), incubated for 10 min on ice, and centrifuged for 10 s at highest speed. The supernatant was discarded and the pellet was resuspended in 100  $\mu$ l cold buffer C (20 mM Hepes-KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF), incubated on ice for 20 min, and centrifuged for 2 min, 4°C, at highest speed. The supernatant was quick frozen at  $-80^{\circ}\text{C}$ . For organs, the above protocol was modified according to Deryckere and Gannon (58). Pieces of tissue, 0.1  $\times$  0.2 cm large, were frozen in liquid nitrogen, broken mechanically with a hammer, and transferred to a 50-ml Falcon tube containing 5 ml cold buffer A (10 mM Hepes-KOH, pH 7.9, at 4°C, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF, 0.6% NP-40). The tissue was homogenized in an Ultrathurrax (Wheaton Scientific, Millville, NJ) for 1 min, transferred to a 15-ml tube, and centrifuged for 30 s at 2,000 rpm, at 4°C, to remove tissue debris. The supernatant was incubated on ice for 10 min and centrifuged for 5 min at 8,000 rpm at 4°C. The supernatant was discarded and the nuclear pellet was resuspended in 100  $\mu$ l buffer B (25% glycerol, 20 mM Hepes-KOH, pH 7.9, at 4°C, 420 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, 2 mM benzamidine, 5 mg/ml leupeptin) and incubated on ice for 20 min. Cellular debris was removed by 2 min of centrifugation at 4°C and the supernatant was quick frozen at  $-80^{\circ}\text{C}$ .

Protein concentrations were determined according to Bradford (59). The following oligonucleotides were synthesized on a Gene Assembler Plus (Pharmacia) and purified on histidine gels: NF $\kappa$ B consensus: 5'-AGTTGAGGGGACTTCCAGGC-3', tissue factor-NF $\kappa$ B: 5'-AGGGTCCCGGAGTTTCTACCGGGA-3'. EMSA were per-

formed as described previously (30). Oligonucleotides were labeled to a specific activity  $> 5 \times 10^7$  cpm/ $\mu$ g DNA using T4-polynucleotide kinase (Promega). Binding of NF $\kappa$ B was performed in 10 mM Hepes, pH 7.5, 0.5 mM EDTA, 100 mM KCl, 2 mM DTT, 2% glycerol, 4% Ficoll 400, 0.25% NP-40, 1 mg/ml BSA (DNase free), and 0.1  $\mu$ g/ $\mu$ l poly dI/dC in a total of 20  $\mu$ l as described essentially by Pahl et al. (60). 1 ng of labeled oligonucleotides ( $\sim 50,000$  cpm Cerenkov) was added to 10  $\mu$ g of nuclear extract and incubated at room temperature for 20 min in the appropriate binding buffer. Protein-DNA complexes were separated from the free DNA probe by electrophoresis through 5% native polyacrylamide gels containing 2.5% glycerol and 0.5 $\times$  TBE. The gels were run at room temperature with 30 mA for  $\sim 2.5$  h. Gels were dried under vacuum on Whatmann D-81 paper (Schleicher and Schüll, Dassel, Germany) and exposed for 12–48 h to Amersham Hyperfilms at  $-80^{\circ}\text{C}$  with intensifying screens. Specificity of binding was ascertained by competition with a 160-fold molar excess of cold consensus oligonucleotides and by characterization with specific polyclonal antibodies (Santa Cruz, Heidelberg, Germany).

#### Densitometric quantification of EMSA autoradiograms

Signals obtained in EMSA were quantitated using a Scan-Pack Personal Densitometer (Pharmacia). The determination of the signal area to be measured and the quantitative evaluation were performed independently by two different investigators (A.B. and T.I.). The mean of both measurements was taken for statistical analysis.

#### Intravenous somatic gene transfer

Intravenous somatic gene transfer was performed as described previously (30, 44). 50  $\mu$ g of plasmid DNA (*pGL<sub>2</sub>-Luc*, control plasmid *pGL<sub>2</sub>-basic*, murine tissue factor expression plasmids, *pXTI*-control vector, *IkB $\alpha$*  or mutated Jun expression plasmids) was mixed with 150  $\mu$ g DOTAP (Boehringer Mannheim Biochemicals, Mannheim, Germany). The first injection was performed 7 d before administration of LPS. The second injection was given 2 d before administration of LPS. Each injection was given via the tail vein. The total volume of the DNA-liposome complex was 250  $\mu$ l.

#### Luc assay

Luc activities were determined as previously described (30, 44). Kidneys were harvested after perfusion with 30 ml PBS, and homogenized with a homogenizer (IKA-Werk; Janke & Kunkel GmbH, Staufen, Germany) for 30 s in lysis buffer (Promega) containing 25 mM Tris, pH 7.8, with H<sub>3</sub>PO<sub>4</sub>; 2 mM EDTA; 2 mM DTT 10% glycerol; 1% Triton X-100. After brief centrifugation, the supernatant was collected and the Luc activity was measured with the Luciferase Assay System (Promega) using a luminometer (LB 9501; Lumat, Berthold, Germany) (61, 62). Data were expressed as Luc activity per gram of tissue.

#### Detection of transfected Luc antigen

**Preparation of kidneys.** For immunocytochemistry, mouse kidneys were harvested after the animal had been perfused extensively with 30 ml of PBS to remove unclotted material. Samples were instantaneously frozen in isopentane, cooled in liquid N<sub>2</sub>. Cryostat sections of mouse kidneys were fixed in acetone for 10 min and then incubated for 5 min in 0.6% H<sub>2</sub>O<sub>2</sub> in methanol for blocking endogenous peroxidase activity.

**Preparation of PBMC.** To perform immunocytochemistry, PBMC were fixed in 4% paraformaldehyde in PBS. After washing with PBS, the endogenous peroxidase activity was blocked by incubating in 3% H<sub>2</sub>O<sub>2</sub> in distilled water for 15 min.

**Detection of Luc antigen.** Before adding the anti-Luc antibody (Promega), the sections were incubated in 10% goat serum Tris-HCl buffer (pH 7.5, 150 mM NaCl, 0.3% Triton X-100) for 30 min at room temperature and then incubated with anti-Luc antibody in Tris-HCl buffer 4°C overnight. An anti-rabbit IgG conjugated with peroxidase was used as second antibody. The color reaction was performed with Sigma Fast DAB substrate (3,3'-diaminobenzidine tetrahydrochloride; Sigma Chemie). Hematoxylin (Sigma Chemie) was used for

counterstaining. Negative controls included omission of the first or second antibodies and substitution of the first antibody by nonspecific antibodies (data not shown).

#### Detection of tissue factor antigen

Sections of mouse kidneys were fixed in 4% paraformaldehyde dissolved in PBS, pH 7.4. After washing two times with PBS, sections were incubated with anti-mouse tissue factor antibodies described earlier (44) for 2 h at room temperature in 100 mM Tris-HCl buffer (pH 7.5, 150 mM NaCl, 0.3% Triton X-100). After washing, sections were incubated with the second antibody (goat anti-rat IgG conjugated with peroxidase) for 1 h at room temperature. Color development was performed with AEC (3-amino-9-ethyl-carbazole; Sigma Chemie) and H<sub>2</sub>O<sub>2</sub> (44, 63). Negative controls included omission of the first or second antibodies and substitution of the first antibody by nonspecific antibodies (data not shown).

#### Immunofluorescence

For immunofluorescence studies, the sections of extensively perfused kidneys (see above) were fixed with acetone for 10 min and incubated with anti-fibrin/fibrinogen antibody (fluorescein conjugate; Cappel Laboratories, West Chester, PA) diluted (1:8) with PBS containing NaN<sub>3</sub> for 45 min at room temperature (44).

#### In situ hybridization

In situ hybridization for tissue factor was performed as previously described (30, 44, 64). Briefly, antisense and sense single-strand cRNAs were synthesized from a mouse tissue factor cDNA fragment (721–1,043 bp) subcloned into pGEM2 (Promega) in the presence of digoxigenin-labeled UTP (Boehringer Mannheim Biochemicals). These probes were characterized using Northern blots (data not shown). The kidney sections were fixed in 4% paraformaldehyde in PBS, pH 7.4, acetylated for 15 min at room temperature in freshly prepared 0.25% acetic anhydride in 0.1 M triethanolamine HCl plus 0.9% NaCl, pH 8.0, and incubated with proteinase K (100 mg/ml) for 10 min. Sections were prehybridized for 2 h at room temperature with hybridization buffer containing 0.6 M NaCl, 1 mM EDTA, 10 mM Tris HCl, pH 7.6, 10% dextran sulfate, 0.25% SDS, 100 mM DTT, 50 µg/ml salmon sperm DNA, 100 µg/ml yeast tRNA, 1× Denhardt's solution, 5% dextran, and 50% (vol/vol) deionized formamide. After prehybridization, hybridization solution containing 5–10 ng cRNA probe was applied to each section, followed by incubation in a moist chamber for 16 h at 37°C. Samples were then incubated with 20 µg/ml RNase A (Boehringer Mannheim Biochemicals) for 30 min at 37°C, followed by washing once with 2× SSC plus 50% formamide for 30 min at room temperature and twice with 0.2× SSC for 30 min at 50°C. For immunological detection, the DIG Nucleic Acid Detection Kit (Boehringer Mannheim Biochemicals) was used according to the manufacturer's instructions. In each section, a consecutive cut was stained as control with the sense riboprobe, which did not show a specific signal (data not shown).

#### Determination of thrombin-antithrombin III complexes (TAT)

1 h before drawing the blood by heart puncture from mice, 200 U heparin (Sigma Chemie) was injected via the tail vein. Plasma was prepared by centrifugation of the blood at 3,000 rpm for 10 min. 50 µl of the supernatant was used to measure TAT. The TAT assay was performed according to the manufacturer's instructions (Behring, Marburg, Germany).

#### Statistical analysis

All values are given as mean, with the bars showing standard deviations. The means of groups were compared by ANOVA using the Newman-Keul's test to correct for multiple comparisons. *P* < 0.05 was considered to be statistically significant. Discrimination analysis was performed with the aid of the program StatView (Abacus Concepts Inc., Berkeley, CA).

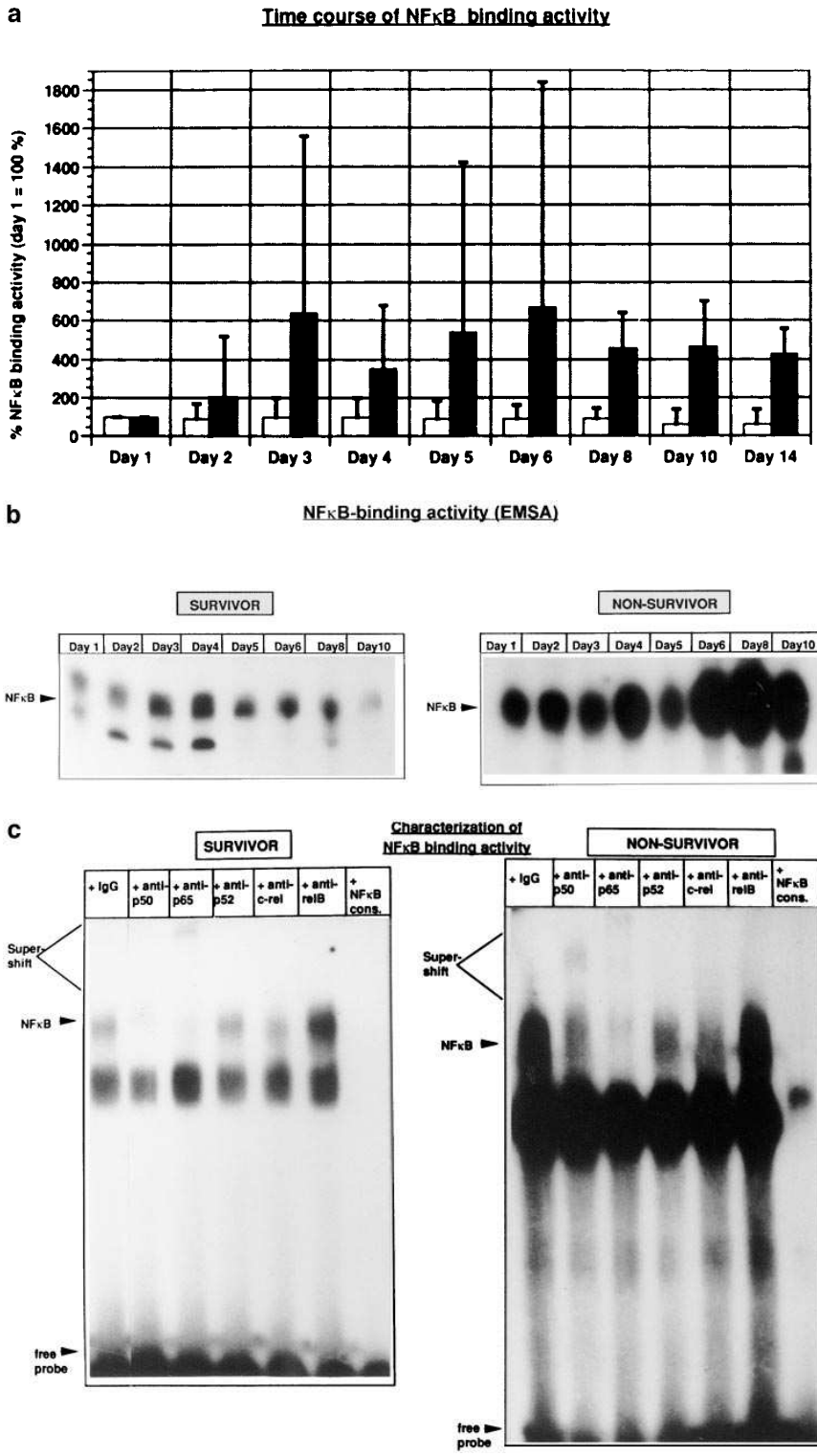
## Results

*NFκB binding activity in nuclear extracts of PBMC from septic patients.* Members of the NFκB family are believed to mediate central events in inflammatory disease. To study the significance of NFκB activation in vivo, 15 septic patients (10 survivors, 5 nonsurvivors) were graded by the APACHE-II score (Table I). NFκB binding activity was determined in nuclear extracts of PBMC by EMSA. Autoradiograms were analyzed by laser densitometry, and the intensity of the gel shift band on day 1 was assigned a value of 100% for comparison with subsequent samples up to day 14 (Table II). All samples from each patient were run simultaneously on the same gel. During the course of the disease, the average NFκB binding activity was lower in survivors than in nonsurvivors (Fig. 2, *a* and *b*). Representative data of one nonsurvivor (patient 12) from days 1 to 10 showed a striking increase in nuclear binding activity (Fig. 2*b*, *right lane*). All patients in whom NFκB binding activity exceeded 200% (compared with day 1) died. This increase did not occur in survivors (Fig. 2*b*, *left*). The proteins binding to the NFκB consensus motif were characterized as members of the NFκB family based on the following criteria: competition with an unlabeled NFκB consensus oligonucleotide (but not AP-1 consensus oligonucleotides, data not shown) and interaction with antibodies directed against proteins of the NFκB family (Fig. 2*c*). Most of the binding activity was characterized as NFκB p50 and NFκB p65, while NFκB p52 and c-rel represented only a minor portion of the binding proteins (Fig. 2*c*). No reduction of NFκB binding activity was observed in the

Table II. NFκB Binding Activity in Patients with Sepsis

Patient No.	% binding activity (day 1 = 100%)								
	D1*	D2	D3	D4	D5	D6	D8	D10	D14
Survivors									
1	100	162	114	170	167	165	61	117	138
2	100	105	126	107	110	117	106	101	106
3	100	128	191	198	150	144	144	57	
4	100	95	70	84	64	101	66	53	47
5	100	75	97	65	7	10	112	31	21
6	100	63	46	114	97	98	96	88	63
7	100	135	150	84	148	128	132	28	120
8	100	66	75	85	86	85	85	88	31
9	100	11	28	29	60	10	51	15	44
10	100	76	95	97	84	86	51	43	31
Nonsurvivors									
11	100	90	1561	331	219	198	581		Death
12	100	130	134	185	141	225	283	213	Death
13	100	154	368						Death
14	100	520	779	680	400	420	350	950	759
15	100	132	350	203	1419	1838	632	230	100

The NFκB signals observed in EMSA of nuclear extracts from PBMC were evaluated by laser densitometry. For EMSA, 10 µg of nuclear extract derived from isolated PBMC was incubated with the NFκB consensus oligonucleotide. The value obtained on day 1 was defined as 100%. All other values were calculated as the percentage of day 1. The data shown represent the mean of two independent measurements. \*D1, day 1, etc.

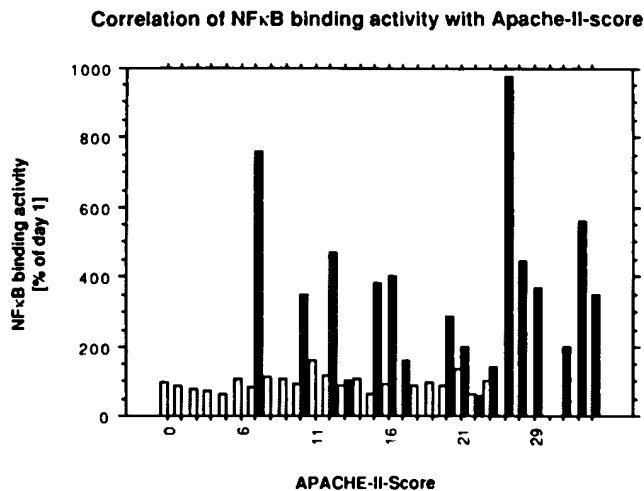


*Figure 2.* Time course of NFκB binding activity in PBMC of septic patients. (a) Time course of NFκB binding activity determined in 10 μg nuclear extracts of PBMC. Each EMSA signal obtained at the time points indicated (Tables I and II) was quantitated by laser densitometry. Signal intensity was determined at each day for all patients surviving ( $n = 10$ ) or not surviving ( $n = 5$ ) and the mean  $\pm$ SD is given for survivors (open bars) and nonsurvivors (filled bars). (b) EMSA of nuclear extract prepared from PBMC isolated from a patient surviving (patient 3) and a patient not surviving (patient 12). 10 μg of nuclear extract was incubated with the NFκB consensus oligonucleotide. The specific NFκB complex is indicated by arrows. (c) Characterization of NFκB binding activity. 10 μg nuclear extract from PBMC of a patient surviving (patient 6, day 1, left) and a patient not surviving (patient 15, day 5, right) was incubated with the NFκB consensus oligonucleotide in the presence of 2.5 μg of the following antibodies: first lane, control IgG; second lane, anti-p50; third lane, anti-p65; fourth lane, anti-p52; fifth lane, anti-c-rel; sixth lane, anti-rel B; seventh lane, binding was competed with a 160-fold excess of unlabeled NFκB consensus oligonucleotide. The inducible NFκB complex is indicated by arrows. A second band, not observed in EMSA without antibodies (compared with b), is probably due to unspecific reactions of the IgG with the NFκB binding motif. Similar results have been obtained with PBMC isolated from other patients.

presence of anti-rel-B antibodies. Using these antibodies, the proteins binding to the NFκB consensus motif were characterized to be identical in survivors and nonsurvivors (Fig. 2c).

The NFκB binding activity was lower in survivors with a lower APACHE-II score than in nonsurvivors with a higher APACHE-II score (Fig. 3). When the variable score was split by outcome, a significantly higher NFκB binding activity was observed in nonsurvivors than in survivors. Discriminate anal-

ysis (Table III) demonstrated that the predictive power of the APACHE-II score was 82%, while the predictive power of PBMC NFκB binding activity was 85%. Both prognostic results did not show significant differences in their predictive power (Newman-Keul's test,  $P > 0.1$ ). Outcome was determined by the severity of the disease and the course of NFκB binding activity. The absolute value of the signal observed on day 1 did not correlate with outcome.



**Figure 3.** Correlation of NFκB binding activity with APACHE-II score. NFκB binding activity was determined with EMSA and quantitated by laser densitometry. The value obtained on day 1 was defined as 100%. The daily values obtained from the 10 patients surviving (open bars) and 5 patients not surviving (filled bars) are shown against the APACHE-II score.

**Mouse LPS model.** The data shown are suggestive of an important role of NFκB activation in the outcome of sepsis. However, the clinical situation is complex and does not allow exact analysis of the role of NFκB activation on survival. Our goal was to determine whether preventing activation of NFκB would influence the outcome of experimentally induced endotoxemia in mice treated with *E. coli* LPS 011:B4 in the presence of galactosamine (51, 52). In this animal model, a time-dependent increase in NFκB binding activity occurred in kidneys and in isolated PBMC (Fig. 4, *a* and *b*), when a lethal dose of LPS was applied. When a nonlethal amount of LPS was injected, only a marginal increase in NFκB binding activity was detected by EMSA (data not shown). Therefore, activation of NFκB in kidneys and in PBMC seems to be comparable in the patient study and in the animal model used.

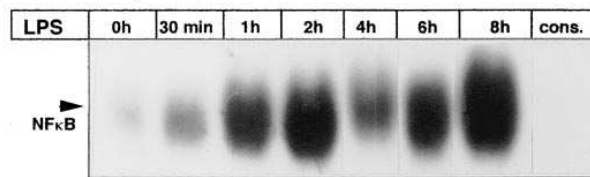
The experimental approach selected to prevent NFκB activation in endotoxemia was intravenous somatic gene transfer with a plasmid overexpressing IκBα (30, 44). To establish an optimal protocol for transfection, somatic gene transfer efficiency experiments were performed with the SV<sub>40</sub>-promoter driven Luc plasmid pGL<sub>2</sub>-Luc and the promoterless control

**Table III. Predictive Power of NFκB Binding Activity and APACHE-II Score**

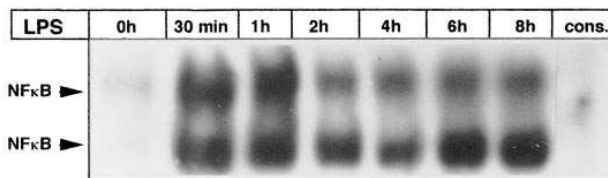
Discriminant score		% of correctly classified cases		
		Survivors (group 1)	Nonsurvivors (group 2)	Survivors and nonsurvivors
APACHE-II score	> 14	83%	79%	82%
NFκB binding activity	> 137	89%	74%	85%

Discriminant analysis of APACHE-II score and NFκB binding activity in PBMC of survivors and nonsurvivors for each analysis point, starting at the day of diagnosis.

**a. Mouse kidney:**



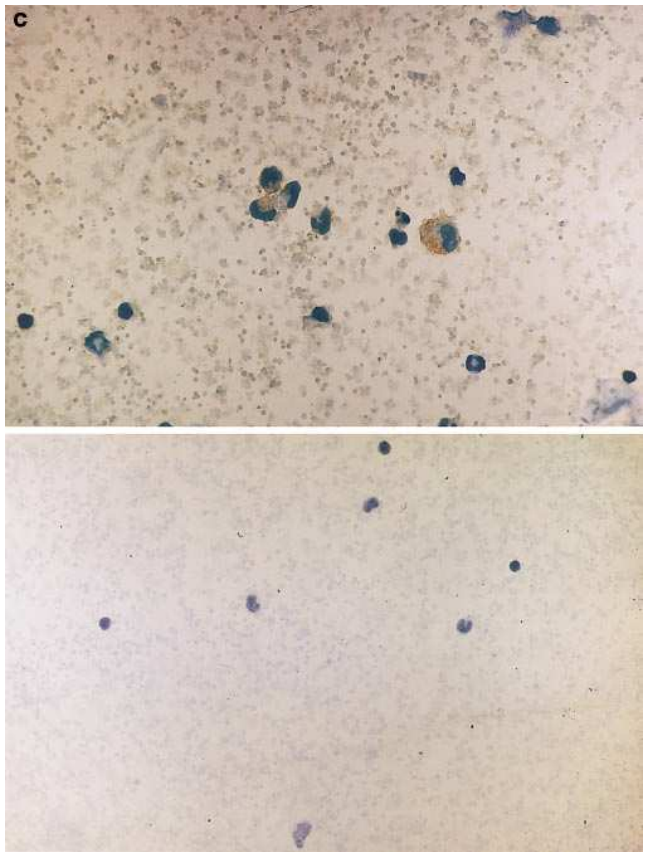
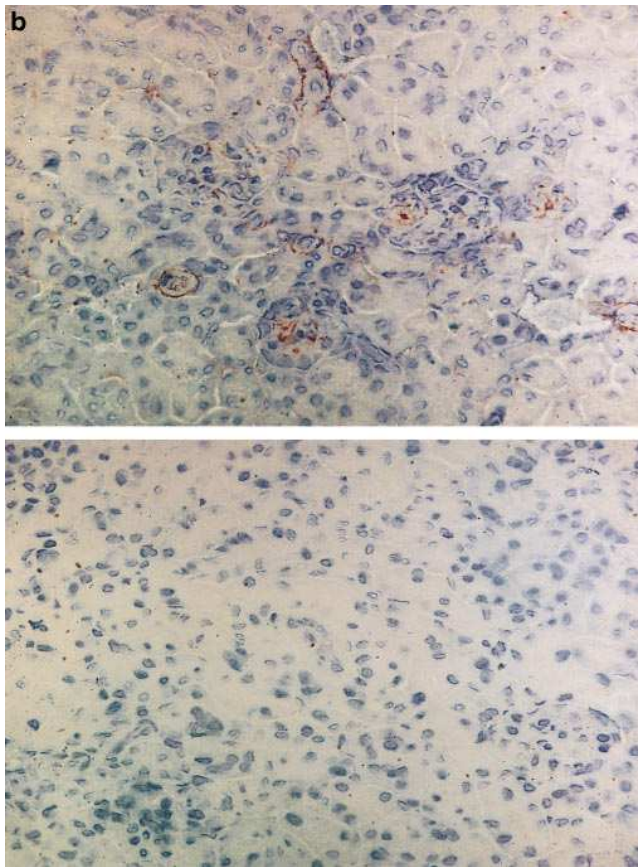
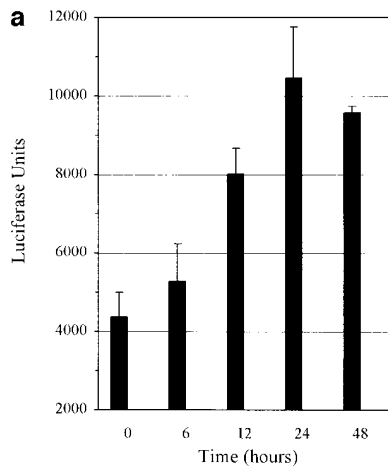
**b. Mouse whole blood:**



**Figure 4.** Time course of NFκB activation in kidneys and PBMC of LPS-treated mice. (*a*) Nuclear extracts were prepared from kidneys of mice that had received LPS (1.75 μg LPS and 15 mg D-galactosamine) for the times indicated. 10 μg of each nuclear extract was analyzed by EMSA for the DNA binding activity of NFκB to an NFκB consensus motif. The inducible NFκB complex is indicated by an arrow. To confirm NFκB binding, nuclear extracts of a mouse that received LPS for 8 h were competed with a 160-fold molar excess of cold consensus NFκB oligonucleotides (*cons.*). (*b*) Mice received LPS as above. For each time point, blood of 10 mice was collected and pooled. PBMC were isolated as described in Methods and nuclear extracts were prepared and assayed for NFκB binding activity. Two inducible NFκB complexes are indicated by arrows. NFκB binding was characterized by competing the binding activity of PBMC nuclear extract obtained 1 h after LPS application with a 160-fold molar excess of cold consensus NFκB oligonucleotides (*cons.*).

vector pGL<sub>2</sub>-basic (Fig. 5). After intravenous administration of the plasmid in DOTAP, reporter gene expression was studied in kidneys, one of the key target tissues of LPS. Luc activity in renal extracts increased steadily after administration of the construct, reaching a maximum between 24 and 48 h (Fig. 5*a*). When the first pulse of plasmid was given 7 d ahead, followed by a second one 5 d later, we found a severalfold higher expression of the reporter plasmid than with a single injection (30, 44). When serial sections of kidney were studied after intravenous somatic gene transfer using pGL<sub>2</sub>-Luc, a significant positive staining (20–35%) was observed in glomerular endothelial cells, arterial and arteriolar endothelial cells, and peritubular capillary endothelial cells (Fig. 5*b*, *left*, and Table IV). Other renal cells, such as tubular epithelial cells, were negative. Only negative staining results were obtained in animals transfected with the control plasmid pGL<sub>2</sub>-basic (Fig. 5*b*, *right*, and Table IV). A similar transfection efficiency was found in isolated PBMC (Fig. 5*c* and Table IV).

**Role of NFκB in LPS-mediated lethality and activation of coagulation.** When mice were treated by intravenous somatic gene transfer with IκBα (or vector pXT1 alone) 5 d and 24 h before administration of LPS, a significant reduction in mortality was observed (Fig. 6). None of the animals died after 24 h. Thus, the mortality caused by LPS in this animal model was in part dependent on NFκB, since inhibition of NFκB by its inhibitor IκBα increased the number of surviving animals. These data confirm that activation of NFκB binding activity in LPS-treated animals might play a central role in setting motion-effect mechanisms relevant to the outcome of endotoxemia.



**Figure 5.** Luc expression after intravenous somatic gene transfer. (a) Time course. Intravenous somatic gene transfer with the luciferase expression plasmid pGL<sub>2</sub>-Luc was performed as described in Methods and Luc expression was determined in renal extracts. The time after pGL<sub>2</sub>-Luc injection and the amount of Luc expression, given as relative Luc units, are shown. The data represent the mean  $\pm$  SD of results obtained from five kidneys derived from five mice in five independent experiments. (b) Luc expression in kidneys. Kidneys were stained with anti-Luc antibodies as described in Methods. Animals were pretreated by intravenous gene transfer with the Luc expression plasmid pGL<sub>2</sub>-Luc (*top*) or the control vector pGL<sub>2</sub>-basic (*bottom*). 10 kidneys from three independent experiments were analyzed. From each kidney, at least 10 sections were stained with similar results. (c) Luc expression in murine PBMC. PBMC were stained with anti-Luc antibodies as described in Methods. Animals were pretreated by intravenous gene transfer with the Luc expression plasmid pGL<sub>2</sub>-Luc (*top*) or the control vector pGL<sub>2</sub>-basic (*bottom*). In each experiment, blood from 10 mice

was collected and pooled, before PBMC were isolated as described in Methods. Isolated PBMC of each experiment were seeded in 10 chamber slides and stained for Luc expression with identical results.

Using the above established protocol of intravenous somatic gene transfer, I $\kappa$ B $\alpha$  was effective in suppressing LPS-mediated NF $\kappa$ B translocation to the nucleus (Fig. 7, *a* and *b*). When animals were pretreated with control vector pGL<sub>2</sub>-basic alone, a strong induction of NF $\kappa$ B binding activity by LPS was observed in EMSA from renal extracts prepared as described in Methods. Pretreatment with I $\kappa$ B $\alpha$  (but not mutated jun) reduced the LPS-mediated NF $\kappa$ B activation. Similar data were obtained using the NF $\kappa$ B consensus motif (Fig. 7*a*) or the NF $\kappa$ B binding region derived from the tissue factor promoter (Fig. 7*b*).

Tissue factor, the central activator of the coagulation mech-

anism, is known to be involved in the lethal effects of LPS. Tissue factor transcription *in vitro* and *in vivo* is under control of NF $\kappa$ B and AP-1 (12–15, 30–34). Treatment of mice with LPS resulted in induction of renal NF $\kappa$ B binding activity to the tissue factor NF $\kappa$ B motif (Fig. 7*b*) and induction of renal tissue factor antigen expression (Fig. 8, *A* and *B*). When intravenous somatic gene transfer with I $\kappa$ B $\alpha$  was performed, an attenuation of LPS-mediated renal tissue factor antigen expression compared with the vector control pXT1 was evident (Fig. 8, *C* vs. *B*). Thus, I $\kappa$ B pretreatment reduced NF $\kappa$ B binding activity to the NF $\kappa$ B motif in the tissue factor promoter and thereby

Table IV. Quantification of Cells Expressing Luc after Intravenous Somatic Gene Transfer

Tissue	Cell type	% positive cells (pGL <sub>2</sub> -Luc expression vector transfected)	% positive cells (pGL <sub>2</sub> -basic control vector transfected)
Kidney	Glomerular endothelial cells	25–35%	0
	Arterial and arteriolar endothelial cells	20–30%	0
	Peritubular capillary endothelial cells	25–30%	0
Blood	PBMC	30–40%	0

Intravenous somatic gene transfer with the pGL<sub>2</sub>-Luc expression plasmid or the promoterless vector pGL<sub>2</sub>-basic was performed and Luc expression was demonstrated using an anti-Luc antibody as described in Methods. Cells not indicated above were luciferase negative. The data were obtained from 10 sections for each of 10 mice. Similar results were obtained from three independent transfections.

reduced expression of the gene product. Accordingly, somatic gene transfer with the sense construct pXTF-s did further increase the amount of tissue factor antigen detected after LPS induction (Fig. 8 D), while pretreatment with the tissue factor antisense construct pXTF-as significantly decreased LPS-mediated tissue factor expression (Fig. 8 E).

Tissue factor has been shown to mediate fibrin deposition in a mouse tumor model (30, 44). Consistent with the data shown in Fig. 7 b and Fig. 8, a reduction in LPS-mediated renal fibrin/fibrinogen deposition was also observed (Fig. 9), when mice were pretreated with IκBα (Fig. 9 C) or the antisense tissue factor plasmid pXTF-as (Fig. 9 E). Extensive in vivo perfusion of the animal with buffer before harvesting the organs ensured that all the unclotted material was removed before staining. Hence, despite the lack of antibody specificity for fibrin, the immunoreactive material is likely to represent in large part fibrin (see also difference of control kidneys vs. LPS-treated kidneys; Fig. 9, A and B) (30, 44).

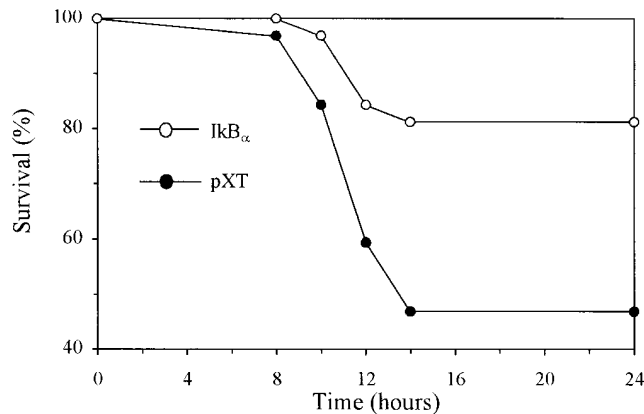


Figure 6. Effect of gene transfer with IκB<sub>α</sub> on LPS-mediated mortality. Survival was determined in mice treated with 1.75 μg LPS and 15 mg D-galactosamine (see Methods). 5 d and 24 h before LPS injection mice were treated with the IκB<sub>α</sub> expression plasmid (*n* = 32) or control vector pXT1 (*n* = 32) by intravenous somatic gene transfer (see Methods). The data give the percentage of surviving animals vs. time. No deaths occurred after 24 h. The difference between IκB<sub>α</sub>-treated animals and animals that had received the control vector pXT1 was statistically significant on the basis of *P* < 0.05. The experiment was repeated three times with identical results.

Decreased tissue factor expression and fibrin/fibrinogen deposition in animals pretreated with IκB was associated with a decrease in plasmatic TAT formation (Fig. 10). Thus, intravenous somatic gene transfer with IκB<sub>α</sub> resulted in decreased expression of tissue factor antigen and tissue factor-dependent activation of the coagulation system.

Next we studied whether renal tissue factor transcription was induced by LPS in vivo in an NFκB-dependent manner. Intravenous somatic gene transfer with the tissue factor promoter-Luc expression plasmid pGLTF<sub>4</sub> (Fig. 1) was performed (Fig. 11). 24 h after injection, a time-dependent increase in Luc activity was measured in renal extracts after LPS administra-

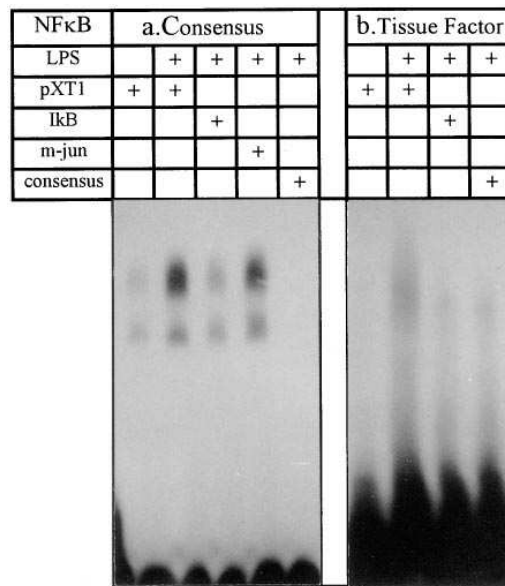
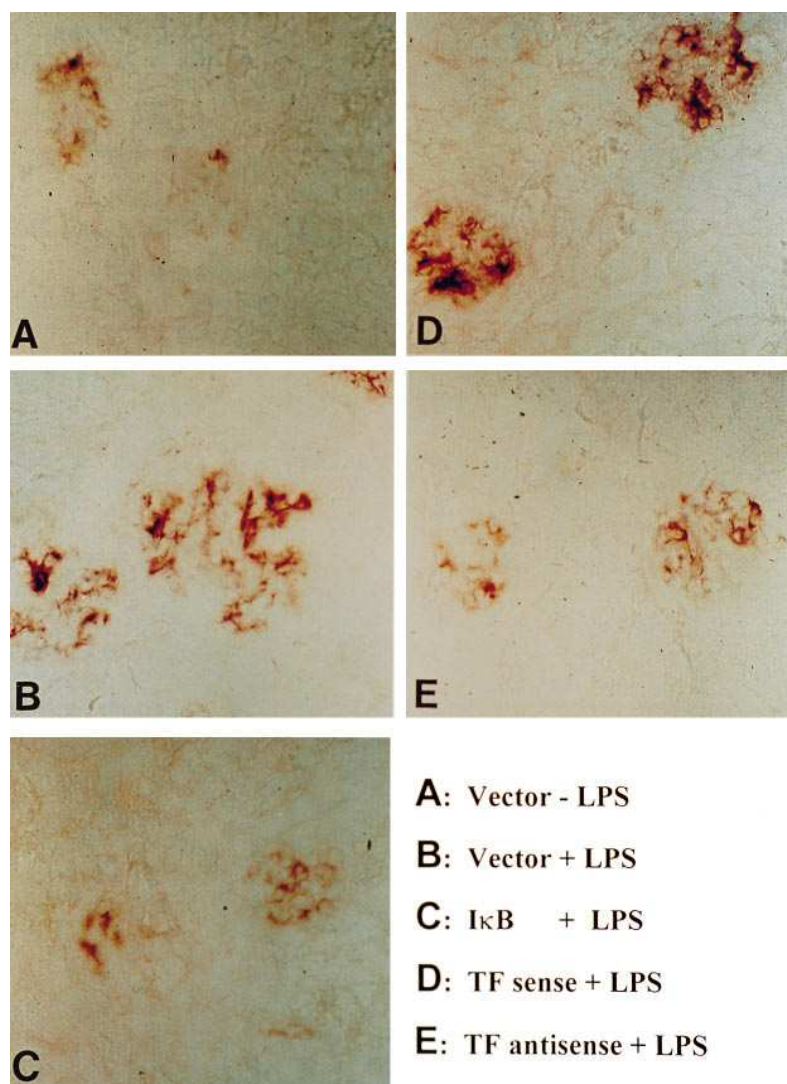


Figure 7. Effect of gene transfer with IκB<sub>α</sub> on LPS-mediated NFκB activation. Nuclear extracts from kidneys were prepared 8 h after injection of 1.75 μg LPS and 15 mg D-galactosamine. 10 μg of nuclear extract was incubated with the NFκB consensus sequence (a) or the NFκB motif derived from the tissue factor promoter (b). Shown is the effect of somatic gene transfer with either the control vector pXT1, IκB<sub>α</sub>, or mutated jun on NFκB binding activity. The specificity of the binding reaction was shown by competition with a 160-fold molar excess of unlabeled NFκB consensus oligonucleotide. The experiment was repeated independently with three kidneys from three animals with similar results.





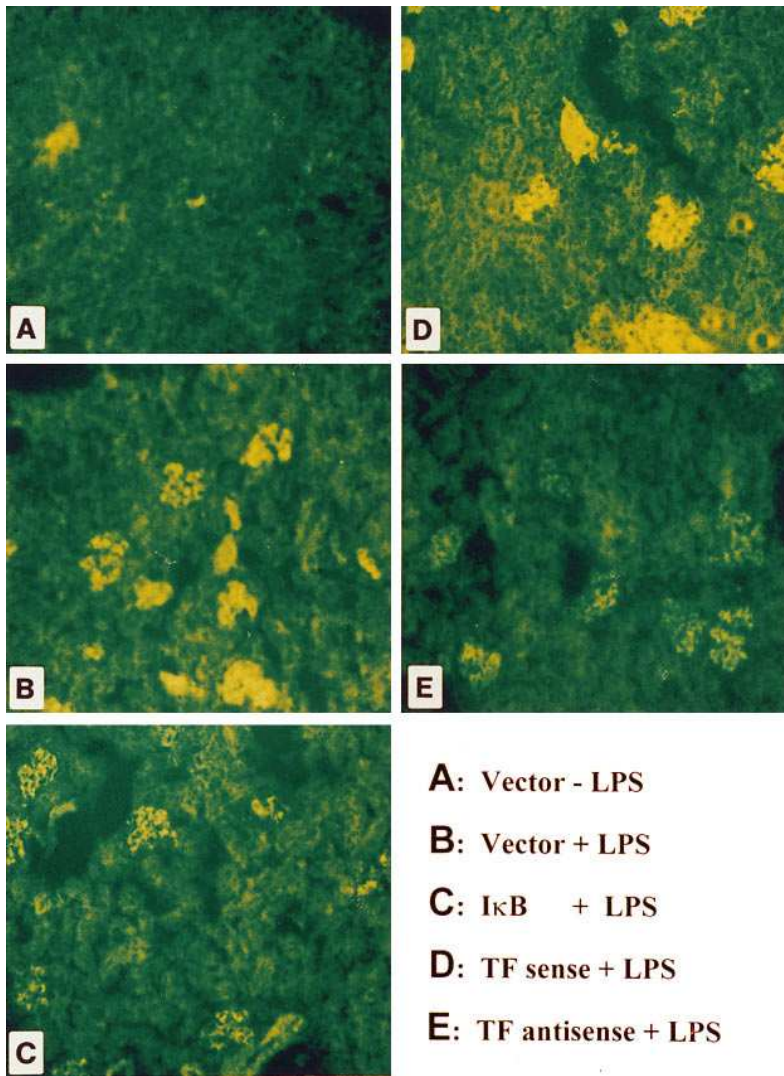
*Figure 8.* Effect of gene transfer with IκBα and tissue factor expression plasmids on the LPS-mediated tissue factor antigen expression. Kidneys were harvested 8 h after treatment with LPS (1.75 μg LPS, 15 mg D-galactosamine) and stained with an anti-tissue factor antibody. Animals were pretreated by intravenous gene transfer with the control vector pXT1, IκBα, or tissue factor expression plasmids. *A*, pXT1 vector; *B*, pXT1 vector plus LPS; *C*, IκBα plus LPS; *D*, tissue factor sense (pXTF-s) plus LPS; and *E*, tissue factor antisense (pXTF-as) plus LPS. Four kidneys from three independent experiments were analyzed. From each kidney, at least five sections were stained with similar results.

tion (Fig. 11 *a*). Using the above established protocol of intravenous gene transfer with IκBα, we studied whether the LPS-mediated increase in transcriptional activity of the tissue factor promoter-Luc plasmid pGLTF<sub>4</sub> was dependent on NFκB. Mice were pretreated with the control vector pXT1 or IκBα together with pGLTF<sub>4</sub>. Compared with pXT1-transfected mice, attenuation of tissue factor promoter activity after LPS treatment was observed in IκBα-pretreated mice (Fig. 11 *b*). Thus, LPS-induced activation of the transcriptional activity of the tissue factor promoter is in part dependent on LPS-mediated NFκB activation.

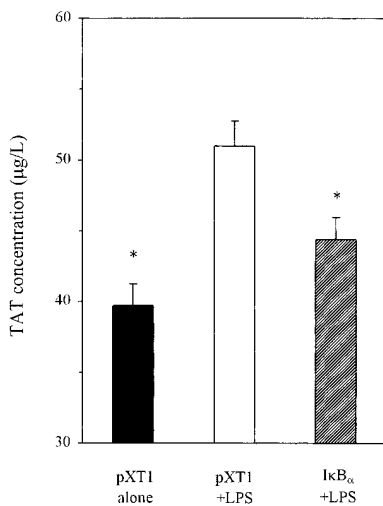
*Role of tissue factor in LPS-mediated lethality and activation of coagulation.* Previous data have shown that neutralization of the tissue factor pathway increases survival and blocks activation of coagulation after LPS (45–47, 65). It is conceivable that preformed cryptic tissue factor is responsible for the LPS-mediated activation of the coagulation mechanism. Another possibility is that the enhanced vascular permeability during sepsis results in exposure of blood components. To study whether NFκB-dependent activation of tissue factor

transcription is involved in the lethal effect of LPS, we performed intravenous somatic gene transfer with tissue factor antisense plasmids and compared the effect on survival with tissue factor sense plasmids or pXT1 vector alone. Intravenous somatic gene transfer with tissue factor cDNA in the antisense direction has been shown to reduce TNFα-mediated tissue factor expression, activation of the coagulation mechanism, and fibrin deposition in a mouse tumor model (44). The tissue factor antisense plasmid pXTF-as significantly increased the number of surviving animals (Fig. 12 *a*), while the tissue factor sense plasmid pXTF-s reduced survival compared with the control vector pXT1. The effect of tissue factor antisense was dependent on the dose of LPS used (Fig. 12 *b*). No effect was present at very high LPS doses.

Treatment with the tissue factor antisense construct reduced LPS-mediated tissue factor transcription (Fig. 13 *F*) and tissue factor antigen induction (Fig. 8 *E*) as well as fibrin/fibrinogen deposition (Fig. 9 *E*) in the kidneys. Furthermore, decreased formation of TAT was observed in tissue factor antisense-pretreated animals (Fig. 14). Thus, induction of tissue



*Figure 9.* Effect of gene transfer with IκBα and tissue factor expression plasmids on LPS-mediated renal fibrin/fibrinogen deposition. Kidneys were harvested 8 h after treatment with LPS (1.75 μg LPS, 15 mg D-galactosamine) and stained with an anti-fibrin/fibrinogen antibody. Animals were pretreated by intravenous gene transfer with the control vector pXT1, IκBα, or tissue factor expression plasmids. *A*, pXT1 vector; *B*, pXT1 vector plus LPS; *C*, IκBα plus LPS; *D*, tissue factor sense (pXTF-s) plus LPS; and *E*, tissue factor antisense (pXTF-as) plus LPS. Four kidneys from three independent experiments were analyzed. From each kidney at least five sections were stained with similar results.



*Figure 10.* Effect of gene transfer with IκBα on LPS-mediated increase in TAT formation. TAT were determined in plasma of mice 8 h after treatment with LPS (1.75 μg LPS and 15 mg D-galactosamine). Mice were pretreated by intravenous somatic gene transfer with the control vector pXT1 alone (black bar,  $n = 5$ ), control vector pXT1 plus LPS (open bar,  $n = 5$ ), or IκBα + LPS (hatched bar,  $n = 5$ ).

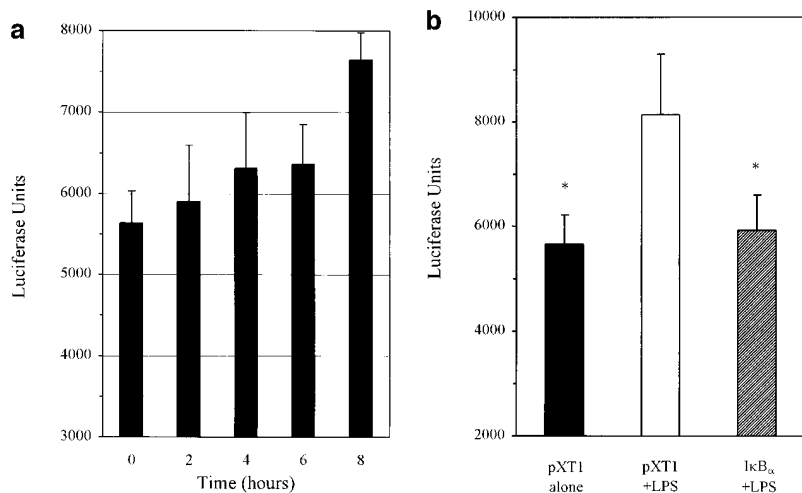
The data represent the mean of three different experiments  $\pm$  SD. The results of pXT1 vs. pXT1/LPS-treated animals and pXT1/LPS vs. IκBα/LPS-treated animals were statistically significant on the basis of  $P < 0.05$ . Statistical significance is indicated by asterisks.

factor transcription and expression is involved in the lethal effect of LPS and activation of coagulation.

## Discussion

Most of the data describing a role of NFκB in inflammatory disease are derived from in vitro studies. We used sepsis and LPS-induced endotoxemia as models to study the role of NFκB activation in vivo. Fatal outcome in patients with sepsis was predicted by an increase in NFκB binding activity PBMC after day 1. All patients with an NFκB binding activity, exceeding 200% of day 1, died. These data support the concept that NFκB activation might be an important event in clinical sepsis. However, the number of patients studied was too small to allow for a final statement. In addition, a correlation between the APACHE-II score and the NFκB binding activity of circulating cells cannot prove a causal relationship even in a larger clinical trial.

To verify a causal relationship between NFκB activation and endotoxemia, intravenous somatic gene transfer with an expression plasmid coding for IκBα (the inhibitor of NFκB) was tested as a tool for analyzing the role of NFκB in a standard mouse model (51, 52), in which the addition of D-galac-

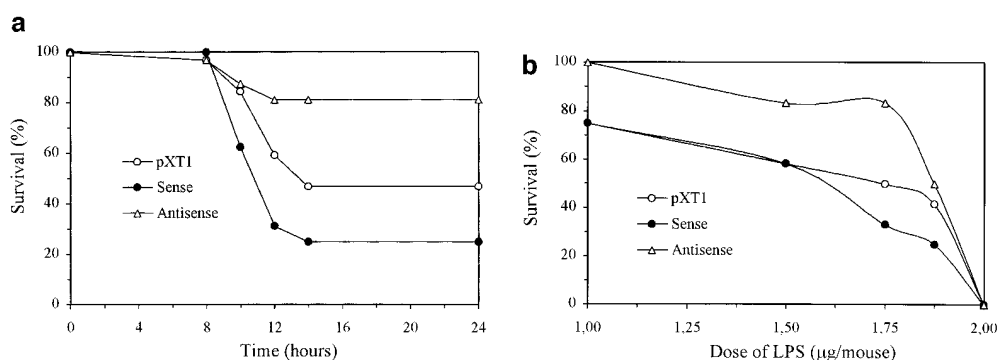


**Figure 11.** Time course of tissue factor promoter activity and the effect of gene transfer with  $\text{I}\kappa\text{B}\alpha$  on the tissue factor promoter activity. (a) Time course. Mice were treated by intravenous somatic gene transfer with the tissue factor promoter-Luc expression plasmid pGLTF4 (Fig. 1). 24 h after the second gene transfer, 1.75  $\mu\text{g}$  LPS and 15 mg D-galactosamine were injected. After LPS injection, renal extracts were prepared for Luc activity assay at the times indicated. The data shown represent the mean  $\pm$  SD of 10 kidneys for each time point, given as relative Luc units. (b) Gene transfer with  $\text{I}\kappa\text{B}\alpha$ . Mice were pretreated by intravenous somatic gene transfer with control vector pXT1 (black bar,  $n = 5$ ), pXT1 plus LPS (open bar,  $n = 5$ ), or  $\text{I}\kappa\text{B}\alpha$  plus LPS (hatched bar,  $n = 5$ ) simultaneously with the tissue factor promoter-Luc expression plasmid pGLTF4. Luc activity was measured in renal extracts 24 h after the second gene transfer and 8 h after adminis-

tration of LPS (1.75  $\mu\text{g}$  LPS and 15 mg D-galactosamine). The data shown represent the mean of the Luc determination performed in 10 kidneys  $\pm$  SD and are expressed as relative Luc units. The results of pXT1 vs. pXT1/LPS-treated animals and pXT1/LPS vs.  $\text{I}\kappa\text{B}\alpha$ /LPS-treated animals were statistically significant on the basis of  $P < 0.05$ . Statistical significance is indicated by asterisks.

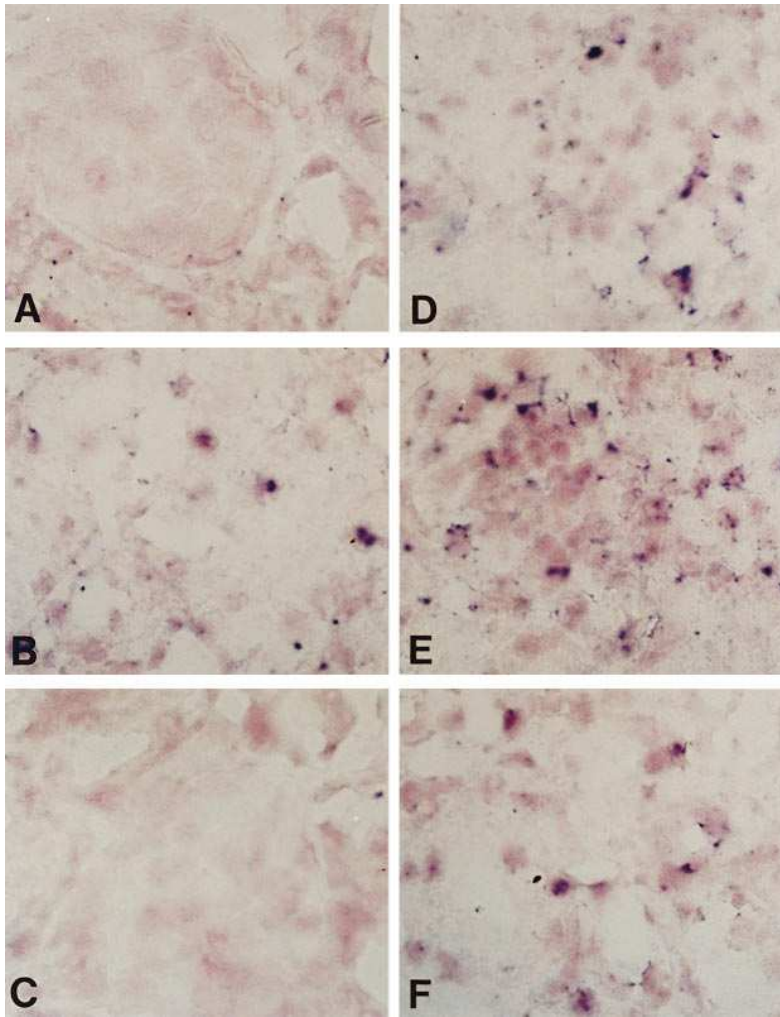
tosamine conditioned the mice to mortality 8–12 h after LPS administration. This model allowed us to clearly define the role of NF $\kappa$ B activation in endotoxemia-mediated lethality. As shown previously (30, 44), intravenous somatic gene transfer can be used to affect biological responses of circulating blood cells and resting cells, including endothelial cells. Intravenous somatic gene transfer with an  $\text{I}\kappa\text{B}\alpha$  expression plasmid reduced LPS-mediated NF $\kappa$ B activation in renal extracts and increased survival after LPS. The experiments provide evidence that NF $\kappa$ B partly mediates LPS-induced mortality. However, they do not prove that the LPS effect itself is dependent only on this transcription factor. The only partial prevention of mortality could be due to several reasons: (a) involvement of other transcription factors; (b) non-transcription-dependent mediators; and (c) a low transfection efficiency by intravenous somatic gene transfer ( $\sim 20$ –40%, Table IV).  $\text{I}\kappa\text{B}\alpha$  preferen-

tially inhibits NF $\kappa$ B p50 and p65. The characterization of the proteins binding to the NF $\kappa$ B consensus motif also revealed the presence of other members of the NF $\kappa$ B family (Fig. 2 c). Thus, a complete inhibition of NF $\kappa$ B cannot be expected by gene transfer with  $\text{I}\kappa\text{B}\alpha$ . However, intravenous somatic gene transfer was at least partially effective as evidenced by the inhibition of NF $\kappa$ B translocation shown in EMSAs of renal extracts (Fig. 7). Therefore, this method may be used to further delineate the possible mechanism of endotoxemia at the level of the transcription factors involved. It has to be noted that an animal model of LPS-mediated lethality reproduces only partly human septicemia. Although the animal model cannot directly be transferred to the clinical situation, it provides a rational basis for the hypothesis that NF $\kappa$ B activation observed in patients has a predictive value in determining the outcome of sepsis.



**Figure 12.** Effect of gene transfer with tissue factor expression plasmids on survival time. (a) The survival of mice treated with LPS (1.75  $\mu\text{g}$  LPS and 15 mg D-galactosamine) was determined. Mice were treated with the tissue factor expression plasmids (Sense,  $n = 32$ ; Antisense,  $n = 32$ ; or pXT1,  $n = 32$ ) 5 d and 24 h before administration of LPS. Data shown represent percent survival vs. time. No deaths occurred after 24 h. The experiment was repeated three times with similar results. The difference between LPS-treated animals that had received the sense tissue factor construct pXTF-s or the control vector pXT1 compared with animals pretreated with the antisense tissue factor plasmid pXTF-as was statistically significant on the basis of  $P < 0.05$ . (b) Dose dependence. Mice were pretreated with the tissue factor expression plasmids (Sense,  $n = 12$ ; Antisense,  $n = 12$ ; or pXT1,  $n = 12$ ) 5 d and 24 h before administration of LPS. Shown is the survival of the 12 animals 24 h after LPS administration. The difference between LPS-treated animals that had received the sense tissue factor construct pXTF-s or the control vector pXT1 compared with animals pretreated with the antisense tissue factor plasmid pXTF-as was statistically significant on the basis of  $P < 0.05$  as long as the LPS dose did not exceed 1.75  $\mu\text{g}$  per mouse.

tioned after 24 h. The experiment was repeated three times with similar results. The difference between LPS-treated animals that had received the sense tissue factor construct pXTF-s or the control vector pXT1 compared with animals pretreated with the antisense tissue factor plasmid pXTF-as was statistically significant on the basis of  $P < 0.05$ . (b) Dose dependence. Mice were pretreated with the tissue factor expression plasmids (Sense,  $n = 12$ ; Antisense,  $n = 12$ ; or pXT1,  $n = 12$ ) 5 d and 24 h before administration of LPS. Shown is the survival of the 12 animals 24 h after LPS administration. The difference between LPS-treated animals that had received the sense tissue factor construct pXTF-s or the control vector pXT1 compared with animals pretreated with the antisense tissue factor plasmid pXTF-as was statistically significant on the basis of  $P < 0.05$  as long as the LPS dose did not exceed 1.75  $\mu\text{g}$  per mouse.



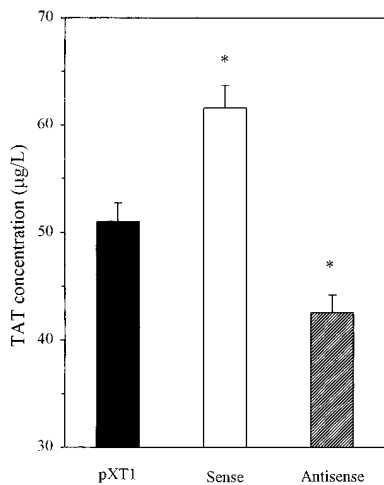
**Figure 13.** Effect of tissue factor expression plasmids on LPS-mediated renal tissue factor transcription. Kidneys were harvested 8 h after treatment with LPS (1.75  $\mu$ g LPS, 15 mg D-galactosamine) and analyzed by in situ hybridization for the presence of tissue factor transcription. Animals were pretreated by intravenous somatic gene transfer with the control vector pXT1 and the tissue factor sense and antisense expression plasmids. *A*, pXT1 vector; *B*, tissue factor sense (pXTF-s); *C*, tissue factor antisense (pXTF-as); *D*, pXT1 vector plus LPS; *E*, tissue factor sense (pXTF-s) plus LPS; *F*, tissue factor antisense (pXTF-s) plus LPS. Four kidneys from three independent experiments were analyzed. From each kidney, at least five sections were stained with similar results.

Gene transfer with  $I\kappa B\alpha$  was not effective when given simultaneously with or after LPS. Thus,  $I\kappa B\alpha$  has to be expressed before LPS is injected, which is compatible with NF $\kappa$ B-dependent gene induction after LPS administration. The hypothesis is that gene transfer has to affect the cell before an amplification limb releases mediators critical in the pathophysiology of endotoxemia. This might explain the lack of effectiveness of TNF $\alpha$  neutralizing antibodies in clinical trials. If this hypothesis is correct, then one has to assume that NF $\kappa$ B activation may not only occur in cells stimulated by LPS, but also in neighboring cells stimulated by mediators released from target cells of LPS. Future studies will have to determine which cells are indirectly affected by LPS. However, since we were able to show that endothelial cells and monocytes/macrophages were transfected by intravenous somatic gene transfer (Fig. 5), these cells might be most probably the critical targets for the action of LPS.

Antibodies against TNF $\alpha$  have been shown to improve survival of animals in endotoxemia models, however, they failed to significantly improve survival in patients with septicemia. An important difference between animal studies and human disease is the time point of intervention. In animal studies, TNF $\alpha$  neutralizing antibodies are applied much earlier in the course of the disease than in humans sepsis, in which patients have been ill for some time before a diagnosis is made. Since

timing of administration and dose of TNF $\alpha$  neutralizing antibodies are critical for efficient reduction of endotoxemia-dependent mortality in animals (66), different timing of TNF $\alpha$  neutralizing antibodies might account for the conflicting results obtained in animal models and clinical trials. Cytokines other than TNF $\alpha$  might also contribute to septicemia-mediated NF $\kappa$ B activation and subsequent gene expression. Cytokine activation of NF $\kappa$ B lasts in vitro for only short time; therefore, we hypothesize that yet unknown mechanisms perpetuate NF $\kappa$ B activation and NF $\kappa$ B-dependent gene expression observed in prolonged inflammatory response. In contrast to the short-lasting NF $\kappa$ B activation induced by TNF $\alpha$ , we observed recently that ligands of the TNF $\alpha$ -inducible cellular receptor RAGE mediate perpetuated NF $\kappa$ B activation (Bierhaus, A., and P.P. Nawroth, unpublished observations). Thus, blocking TNF $\alpha$  by neutralizing antibodies might only in part inhibit LPS-dependent NF $\kappa$ B activation and therefore fail to inhibit pathways of NF $\kappa$ B activation downstream of TNF $\alpha$ . Furthermore, it has been reported that TNF $\alpha$  antibodies specifically inhibited fibrinolysis and thereby enhanced the risk for microvascular thrombosis (23). Thus, in the course of human sepsis side effects of TNF $\alpha$  neutralizing antibodies might cause long-term complications and mortality, which are not evident in time-defined animal models.

Tissue factor has been described previously as an impor-



**Figure 14.** Effect of tissue factor on LPS-induced TAT formation. TAT were determined in mice 8 h after treatment with 1.75 µg LPS and 15 mg D-galactosamine. Mice were pretreated by intravenous somatic gene transfer with control vector pXT1 (black bar,  $n = 5$ ), tissue factor sense (pXTF-s; open bar,  $n = 5$ ), or tissue factor antisense (pXTF-as; hatched bar,  $n = 5$ ). The data represent the mean of three independent

experiments  $\pm$  SD. The difference between LPS-treated animals that had received the sense tissue factor plasmid pXTF-s or the control vector pXT1 compared with animals pretreated with the antisense tissue factor plasmid pXTF-as was statistically significant on the basis of  $P < 0.05$ .

tant mediator of LPS-mediated activation of the coagulation system and of mortality in sepsis (45–47, 65). Recently, it was also shown that TNF $\alpha$ -induced fibrin formation in tumor capillaries is in part dependent on NF $\kappa$ B-mediated tissue factor induction (30, 44). Therefore, tissue factor might well be an important target of LPS-induced NF $\kappa$ B activation in sepsis and endotoxemia. The data shown suggest that not only mobilization of cryptic tissue factor, but also de novo NF $\kappa$ B-dependent transcription of tissue factor mediate TAT formation and fibrin deposition that in part contribute to the lethal effects of LPS. Thus, NF $\kappa$ B activation plays an important role in tissue factor-mediated “consumption coagulopathy” (67). I $\kappa$ B $\alpha$  and tissue factor antisense expression plasmids reduced renal tissue factor transcription after LPS administration (in situ hybridization, Fig. 13). Therefore, the mechanism involved in LPS-induced activation of the coagulation mechanism resembles TNF $\alpha$ -mediated fibrin deposition in capillaries of MethA-sarcomas (30, 44).

Tissue factor is not the only mediator involved in LPS-mediated mortality, since antisense tissue factor prevented only part of the LPS effect. One explanation is the rather low transfection efficiency of intravenous somatic gene transfer. Another explanation is that other genes controlled by NF $\kappa$ B, such as cytokines, endothelin-1, leukocyte adhesion molecules, and others, are also involved in endotoxemia. Moreover, mediators not dependent on protein synthesis might also be involved. Thus, the patient study and the animal study complement each other in demonstrating that LPS-mediated activation of the transcription factor NF $\kappa$ B plays a central role in human disease and in an animal model of endotoxemia. Intravenous somatic gene transfer with I $\kappa$ B $\alpha$  can be used to analyze the contribution of NF $\kappa$ B to endotoxemia. Further applications of this approach may include ischemia-reperfusion and transplant rejection.

## Acknowledgments

We thank Nigel Mackman (Scripps Research Institute, La Jolla, CA) for providing the human tissue factor promoter plasmid pL4, Daniel

Nathans for providing the mouse tissue factor cDNA, Thomas Luther (Dresden, Germany) for providing anti-tissue factor antibodies, Patrick A. Baeuerle for providing the I $\kappa$ B $\alpha$  expression plasmid, and Dirk Bohmann for providing the mutated jun expression plasmid.

This work was supported by a grant from the Deutsche Forschungsgemeinschaft (P. Nawroth). P. Nawroth was supported by a Heisenberg Stipend from DFG and by the Schilling-Stiftung. D. Stern was supported by grants HL-42833 and HL-42507. H. Böhrer was supported by a grant from Immuno.

## References

- Centers for Disease Control (CDC). 1990. *Morb. Mortal. Wkly. Rep.* 39: 31–34.
- Zuckerman, S.H., G.F. Evans, and L. Guthrie. 1991. Transcriptional and post-transcriptional mechanisms involved in the differential expression of LPS-induced IL-1 and TNF mRNA. *Immunology*. 73:460–465.
- Mohri, M., D.R. Spriggs, and D. Kufe. 1990. Effects of lipopolysaccharide on phospholipase A2 activity and tumor necrosis factor expression in HL-60 cells. *J. Immunol.* 144:2678–2682.
- Mai, U.E., G.I. Perez-Perez, L.M. Wahl, S.M. Wahl, M.J. Blaser, and P.D. Smith. 1991. Soluble surface proteins from *Helicobacter pylori* activate monocytes/macrophages by lipopolysaccharide-independent mechanism. *J. Clin. Invest.* 87:894–900.
- Ray, A., M. Hannink, and B.K. Ray. 1995. Concerted participation of NF- $\kappa$ B and C/EBP heteromers in lipopolysaccharide induction of serum amyloid A gene expression in liver. *J. Biol. Chem.* 270:7365–7374.
- Geng, Y., E. Gulbins, A. Altman, and M. Lotz. 1994. Monocyte deactivation by interleukin 10 via inhibition of tyrosine kinase activity and the Ras signaling pathway. *Proc. Natl. Acad. Sci. USA.* 91:8602–8606.
- Tebo, J.M., W. Chaoqun, Y. Ohmori, and T.A. Hamilton. 1994. Murine inhibitory protein-kappa B alpha negatively regulates kappa B-dependent transcription in lipopolysaccharide-stimulated RAW 264.7 macrophages. *J. Immunol.* 153:4713–4720.
- Tran-Thi, T.-A., K. Decker, and P.A. Baeuerle. 1995. Differential activation of transcription factor NF $\kappa$ B and AP-1 in rat liver macrophages. *Hepatology*. 22:613–619.
- Read, M.A., S.R. Cordle, R.A. Veach, C.D. Carlisle, and J. Hawiger. 1993. Cell-free pool of CD14 mediates activation of transcription factor NF- $\kappa$ B by lipopolysaccharide in human endothelial cells. *Proc. Natl. Acad. Sci. USA.* 90:9887–9891.
- Ding, A., S. Hwang, H.M. Lander, and Q.W. Xie. 1995. Macrophages derived from C3H/HeJ (Lpsd) mice respond to bacterial lipopolysaccharide by activating NF- $\kappa$ B. *J. Leukocyte Biol.* 57:174–179.
- Zuckerman, S.H., and G.F. Evans. 1992. LPS tolerance: in vivo regulation of tumor necrosis factor and interleukin-1 synthesis is at the transcriptional level. *Cell. Immunol.* 140:513–519.
- Parry, G.C.N., and N. Mackman. 1995. Transcriptional regulation of tissue factor expression in human endothelial cells. *Arterioscler. Thromb.* 15: 612–621.
- Bierhaus, A., C.J. Hemmer, N. Mackman, R. Kutob, R. Ziegler, M. Dietrich, and P.P. Nawroth. 1995. Antiparasitic treatment of patients with *P. falciparum* malaria reduces the ability of patient serum to induce tissue factor by decreasing NF $\kappa$ B activation. *Thromb. Haemostasis.* 73:39–48.
- Oeth, P.A., G.C.N. Parry, C. Kunsch, P. Nantermet, C.A. Rosen, and N. Mackman. 1994. Lipopolysaccharide induction of tissue factor gene expression in monocytic cells is mediated by binding of c-Rel/p65 heterodimers to a kappaB-like site. *Mol. Cell. Biol.* 14:3772–3781.
- Parry, G.C.N., and N. Mackman. 1994. A set of inducible genes expressed by activated human monocytic and endothelial cells contain kappaB-like sites that specifically bind c-Rel-p65 heterodimers. *J. Biol. Chem.* 269:20823–20825.
- Moler, K.M., P.R. Sleath, J.N. Fitzner, D.P. Cerretti, M. Alderson, S.S. Kerwar, D.S. Torrance, C. Otten-Evans, T. Greenstreet, and K. Weerawarna. 1994. Protection against a lethal dose of LPS by an inhibitor of tumor necrosis factor processing. *Nature (Lond.)*. 370:218–220.
- Torti, F.M., B. Dieckmann, B. Beutler, A. Cerami, and G.M. Ringold. 1985. A macrophage factor inhibits adipocyte gene expression: an in vitro model of cachexia. *Science (Wash. DC)*. 229:867–869.
- Tracey, K.J., B. Beutler, S.G. Lowry, J. Merryweather, S. Wolpe, I.W. Milsark, R.J. Hariri, T.J. Fahey III, A. Zentella, J.D. Albert, et al. 1986. Shock and tissue injury induced by recombinant human cachectin. *Science (Wash. DC)*. 234:470–474.
- Tracey, K.J., Y. Fong, D.G. Hesse, K.R. Monogue, A.T. Lee, G.C. Kuo, S.T. Lowry, and A. Cerami. 1987. Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteremia. *Nature (Lond.)*. 330:662–664.
- Ashkenazi, A., S.A. Marsters, D.J. Capon, S.M. Chamow, I.S. Figari, D. Pennica, D.V. Goeddel, M.A. Palladino, and D.H. Smith. 1991. Protection against endotoxic shock by a tumor necrosis factor receptor immunoadhesin. *Proc. Natl. Acad. Sci. USA.* 88:10535–10539.

21. Miethke, T., C. Wahl, K. Heeg, B. Echtenacher, P.H. Kramer, and H. Wagner. 1992. T cell-mediated lethal shock triggered in mice by the superantigen staphylococcal enterotoxin B: critical role of tumor necrosis factor. *J. Exp. Med.* 175:91-98.
22. Pfeffer, K., T. Matsuyama, T.M. Kundig, A. Wakeham, K. Kishihara, A. Shahinian, K. Wiegmann, P.S. Ohashi, M. Kronke, and T.W. Mak. 1993. Mice deficient for the 55 kd tumor necrosis factor receptor are resistant to endotoxic shock, yet succumb to *L. monocytogenes* infection. *Cell*. 73:457-467.
23. Van der Poll, T., M. Levi, S.J.H. Van Deventer, H. ten Cate, B.L. Haagmans, B.J. Biemond, H.R. Büller, C.E. Hack, and J.W. ten Cate. 1994. Differential effects of anti-tumor necrosis factor monoclonal antibodies on systemic inflammatory responses in experimental endotoxemia in chimpanzees. *Blood*. 83:446-451.
24. Wheeler, A.P., and G.R. Bernard. 1996. Applications of molecular biology and biotechnology: antibody therapy of sepsis. *J. Crit. Care*. 11:77-94.
25. Reinhart, K., C. Wiegand-Lohnert, F. Grimminger, M. Kaul, S. Withington, and D. Treacher. 1996. Assessment of the safety and efficacy of the monoclonal anti-tumor necrosis factor antibody-fragment, MAK 195F, in patients with sepsis and septic shock: a multicenter, randomized, placebo-controlled, dose-ranging study. *Crit. Care Med.* 24:733-742.
26. von-der-Mohlen, M.A., T. van-der-Poll, J. Jansen, M. Levi, and S.J. van-Deventer. 1996. Release of bactericidal/permeability-increasing protein in experimental endotoxemia and clinical sepsis. Role of tumor necrosis factor. *J. Immunol.* 156:4969-4973.
27. Edgington, T.S., N. Mackman, S.T. Fan, and W. Ruf. 1992. Cellular immune and cytokine pathways resulting in tissue factor expression and relevance to septic shock. *Nouv. Rev. Fr. Hematol.* 34:S15-S37.
28. Mackman, N., M.S. Sawday, M.R. Keeton, and D.J. Loskutoff. 1993. Murine tissue factor gene expression in vivo. Tissue and cell specificity and regulation by lipopolysaccharide. *Am. J. Pathol.* 143:76-84.
29. Colucci, M., G. Balconi, R. Lorenzet, A. Pietra, D. Locati, M.B. Donati, and N. Semeraro. 1983. Cultured human endothelial cells generate tissue factor in response to LPS. *J. Clin. Invest.* 71:1893-1896.
30. Bierhaus, A., Y. Zhang, Y. Deng, N. Mackman, P. Quehenberger, M. Haase, T. Luther, M. Müller, H. Böhrer, J. Greten, et al. 1995. Mechanism of the tumor necrosis factor  $\alpha$ -mediated induction of endothelial tissue factor. *J. Biol. Chem.* 270:26419-26432.
31. Moll, T., M. Czyz, H. Holzmüller, R. Hofer-Warbinek, E. Wagner, H. Winkler, F.H. Bach, and E. Hofer. 1995. Regulation of the tissue factor promoter in endothelial cells. Binding of NF kappa B-, AP-1-, and Sp1-like transcription factors. *J. Biol. Chem.* 270:3849-3857.
32. Mackman, N. 1995. Regulation of the tissue factor gene. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 9:883-889.
33. Mackman, N. 1994. Protease inhibitor blocks lipopolysaccharide induction of tissue factor gene expression in human monocytic cells by preventing activation of c-Rel/p65 heterodimers. *J. Biol. Chem.* 269:26363-26367.
34. Donovan-Peluso, M.D., L.D. George, and C.A. Hassett. 1994. Lipopolysaccharide induction of tissue factor expression in THP-1 monocytic cells. *J. Biol. Chem.* 269:1361-1369.
35. Nawroth, P.P., and D.M. Stern. 1986. Modulation of endothelial cell hemostatic properties by TNF. *J. Exp. Med.* 164:740-745.
36. Bevilacqua, M., J. Pober, G. Majeau, W. Fiers, R. Cotran, and M. Gimbrone. 1986. Recombinant TNF induces procoagulant activity in endothelium. *Proc. Natl. Acad. Sci. USA.* 83:4533-4537.
37. Nawroth, P.P., D. Handley, C.T. Esmon, and D.M. Stern. 1986. Interleukin 1 induces endothelial cell procoagulant while suppressing cell surface anticoagulant activity. *Proc. Natl. Acad. Sci. USA.* 83:3460-3464.
38. Conway, E.M., B. Ronald, D.R. Robert, and H.K. William. 1989. Tumor necrosis factor enhances expression of tissue factor mRNA in endothelial cells. *Thromb. Res.* 53:231-241.
39. Nawroth, P.P., D. Handley, G. Matsueda, R.D. Waal, H. Gerlach, D. Blohm, and D. Stern. 1988. Tumor necrosis factor/cachectin-induced intravascular fibrin formation in meth-A fibrosarcomas. *J. Exp. Med.* 168:637-647.
40. Lyberg, T., K.S. Galdal, S.A. Evensen, and H. Prydz. 1983. Cellular cooperation in endothelial cell thromboplastin synthesis. *Br. J. Haematol.* 53:85-95.
41. Carson, S.D., and R.J. Donald. 1990. Consecutive enzyme cascades: complement activation at the cell surface triggers increased tissue factor activity. *Blood*. 76:361-367.
42. Tannenbaum, S.H., F. Rena, and B.C. Douglas. 1986. Antibody and immune complexes induce tissue factor production by human endothelial cells. *J. Immunol.* 137:1532-1537.
43. Edwards, R.L., and F.R. Rickles. 1980. The role of human T cells (and T cell products) for monocyte tissue factor generation. *J. Immunol.* 125:606-609.
44. Zhang, Y.M., Y.D. Deng, T. Wendt, B. Liliensiek, A. Bierhaus, J. Greten, W. He, B.S. Chen, V. Hach-Wunderle, R. Waldherr, et al. 1996. Intravenous somatic gene transfer with antisense-tissue factor restores blood flow by reducing tumor necrosis factor induced tissue factor expression and fibrin deposition in mouse Meth-A sarcomas. *J. Clin. Invest.* 97:2213-2224.
45. Warr, T.A., L.V.M. Rao, and S.I. Rapaport. 1990. Disseminated intravascular coagulation in rabbits induced by administration of LPS or tissue factor: effect of anti-tissue factor antibodies and measurement of plasma extrinsic pathway inhibitor activity. *Blood*. 75:1481-1489.
46. Levi, M., H. ten Cate, K.A. Bauer, T. van der Poll, T.S. Edgington, H.R. Büller, S.J.H. Deventer, C.E. Hack, J. Wouter ten Cate, and R.D. Rosenberg. 1994. Inhibition of LPS-induced activation of coagulation and fibrinolysis by pentoxifylline or by a monoclonal anti-tissue factor antibody in chimpanzees. *J. Clin. Invest.* 93:114-120.
47. Taylor, F.B., Jr., A. Chang, W. Ruf, J.H. Morrissey, L. Hinshaw, R. Catlett, K. Blick, and T.S. Edgington. 1991. Lethal *E. coli* septic shock is prevented by blocking tissue factor with a monoclonal antibody. *Circ. Shock*. 33:127-134.
48. Biemond, B.J., M. Levi, H. ten Cate, H.R. Soule, L.D. Morris, D.L. Foster, C.A. Bogowitz, T. Van der Poll, and H.R. Büller. 1995. Complete inhibition of LPS-induced coagulation activation in chimpanzees with a monoclonal Fab fragment against factor VII/VIIa. *Thromb. Haemostasis.* 73:223-230.
49. Fiedler, V.B., I. Loof, E. Sander, V. Voehringer, C. Galanos, and M.A. Fournel. 1992. Monoclonal antibody to tumor necrosis factor-alpha prevents lethal endotoxin sepsis in adult rhesus monkeys. *J. Lab. Clin. Med.* 120:574-588.
50. Ryan, J., J. Brett, P. Tijburg, R.R. Bach, W. Kisiel, and D. Stern. 1992. Tumor necrosis factor  $\alpha$  induced endothelial tissue factor is associated with sub-endothelial matrix vesicles but is not expressed on the apical surface. *Blood*. 80:966-974.
51. Galanos, C., M.A. Freudenberg, and W. Reutter. 1979. Galactosamine-induced sensitization to the lethal effects of LPS. *Proc. Natl. Acad. Sci. USA.* 76:5939-5943.
52. Lehmann, V., M.A. Freudenberg, and C. Galanos. 1987. Lethal toxicity of lipopolysaccharide and tumor necrosis factor in normal and D-galactosamine-treated mice. *J. Exp. Med.* 165:657-663.
53. Hartzell, S., K. Ryder, A. Lanahan, L.F. Lau, and D. Nathans. 1989. A growth factor-responsive gene of murine BALB/c 3T3 cells encodes a protein homologous to human tissue factor. *Mol. Cell. Biol.* 9:2567-2573.
54. Mackman, N., B.J. Fowler, T.S. Edgington, and J.H. Morrissey. 1990. Functional analysis of the human tissue factor promoter and induction by serum. *Proc. Natl. Acad. Sci. USA.* 7:2254-2258.
55. Nolan, G.P., S. Ghosh, H.C. Liou, P. Tempst, and D. Baltimore. 1991. DNA binding and I kappa B inhibition of the cloned p65 subunit of NF-kappa B, a Rel-related polypeptide. *Cell*. 64:961-969.
56. Bohmann, D., and R. Tjian. 1989. Biochemical analysis of transcriptional activation by Jun: differential activity of c- and v-Jun. *Cell*. 59:709-717.
57. Andrews, N.C., and D.V. Faller. 1991. A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. *Nucl. Acids Res.* 19:2499.
58. Deryckere, F., and F. Gannon. 1994. A one-hour miniprep technique for extraction of DNA-binding proteins from animal tissues. *Biotechniques*. 16:405.
59. Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.
60. Pahl, H.L., and P.A. Baeuerle. 1995. Expression of influenza virus hemagglutinin activates transcription factor NF-kappa B. *J. Virol.* 69:1480-1484.
61. de Wet, J.R., K.V. Wood, M. DeLuca, D.R. Helinski, and S. Subramani. 1987. Firefly luciferase gene: structure and expression in mammalian cells. *Mol. Cell. Biol.* 7:725-737.
62. Alam, J., and J.L. Cook. 1990. Reporter genes: application to the study of mammalian gene transcription. *Anal. Biochem.* 188:245-256.
63. Larsson, L.I. 1989. Immunocytochemistry: Theory and Practice. CRC Press, Inc., Boca Raton, FL. 95-96.
64. Zhang, Y.M., S. Bachmann, C. Hemmer, J. van Lunzen, A. von Stemm, P. Kern, M. Dietrich, R. Waldherr, and P.P. Nawroth. 1994. Vascular origin of Kaposi's sarcoma: expression of leukocyte adhesion molecule-1, thrombomodulin, and tissue factor. *Am. J. Pathol.* 144:51-59.
65. Creasey, A.A., A.C.K. Chang, L. Feigen, T.C. Wun, F.B. Taylor, Jr., and L.B. Hinshaw. 1993. Tissue factor pathway inhibitor reduces mortality from *Escherichia coli* septic shock. *J. Clin. Invest.* 91:2850-2860.
66. O'Riordain, M.G., D.S. O'Riordain, R.G. Molloy, J.A. Mannick, and M.L. Rodrick. 1996. Dosage and timing of anti-TNF-alpha antibody treatment determine its effect of resistance to sepsis after injury. *J. Surg. Res.* 64:95-101.
67. Lasch, H.G., D.C. Heene, K. Huth, and W. Sandritter. 1967. Pathophysiology, clinical manifestation and theory of consumption coagulopathy. *Am. J. Cardiol.* 20:381-391.