Role of NFκB in the Mortality of Sepsis

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Abstract

Binding activity for nuclear factor kappa B (NFκB) 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 NFκB binding activity during the observation period ($P < 0.001$). The increase in NFκB binding activity was comparable to the APACHE-II score as a predictor of outcome. Intravenous somatic gene transfer with an expression plasmid coding for IκBα was used to investigate the role of members of the NFκB family in a mouse model of endotoxemia. In this model, increased NFκB binding activity was present after injection of LPS. Intravenous somatic gene transfer with IκBα given before LPS attenuated renal NFκB 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 NFκB, was induced by LPS. Somatic gene transfer with a reporter plasmid containing the functional tissue factor promoter demonstrated NFκB-dependent stimulation by LPS. Intravenous somatic gene transfer with IκBα 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 • NFκB • 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 intact 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 (NFκB)[1]-regulatory DNA sequences. Members of the NFκB 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 NFκB family in vivo is missing. Two particular gene products with a central role in the septic response are TNFα and tissue factor (10–15).

TNFα 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 TNFα-related mechanisms to tissue injury is supported by observations that inhibition of TNFα processing to the active form, passive immunization with neutralizing anti-TNFα antibodies, or gene deletion of the 55-kD TNFα receptor increase survival in animal models of endotoxemia (16–23). However, clinical studies using TNFα-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 NFκB site (12–15, 30–34) and TNFα 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 TNFα and tissue factor expression in vivo is supported by experiments showing that neutralization of TNFα reduces activation of coagulation in vivo (49). However, it remains unclear whether tissue factor expression in vivo depends on NFκB-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

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1. *Abbreviations used in this paper:* EMSA, electrophoretic mobility shift assay; Luc, luciferase; NFκB, 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 $pGLTF_4$-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_2-Luc (Promega, Heidelberg, Germany), from which the SV_{40} promoter was removed (Fig. 1). The promoterless Luc-control vector pGL_2-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 2×10^6 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.). 10^6 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). 2×10^6 isolated PBMC were lysed in 400 μl cold buffer A (10 mM Hepes-KOH, pH 7.9, at 4°C, 1.5 mM $MgCl_2$, 10 mM KCl,

Table I. Description of Patients

				APACHE-II score								
Patient No.	Age	Sex	Cause of sepsis	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‡	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. ‡ *AHNP*, acute hemorrhagic necrotizing pancreatitis.

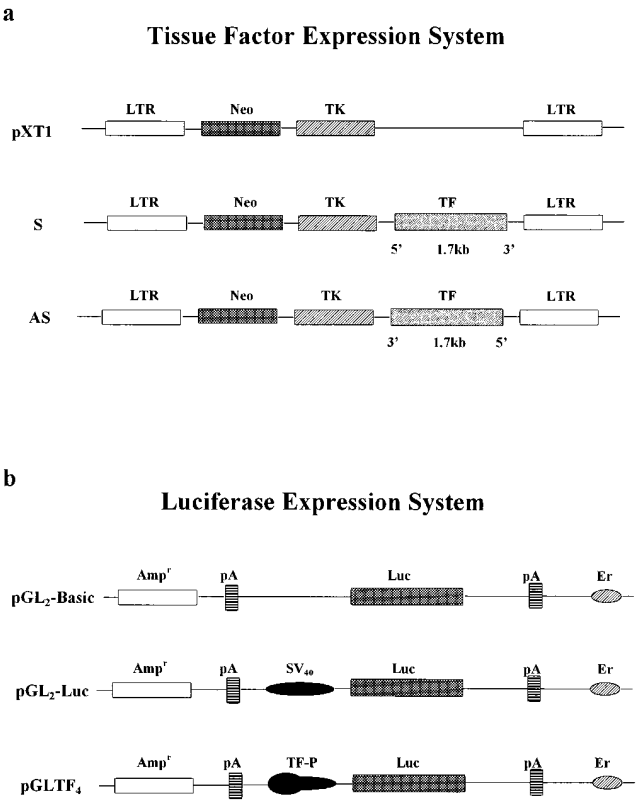

Figure 1. Scheme of expression plasmids. (*a*) Tissue factor expression system. *pXT1*, vector plasmid; *S*, sense tissue factor expression plasmid pXTF-s; *AS*, antisense tissue factor expression plasmid pXTF-as; *LTR*, Moloney murine leukemia virus long terminal repeats; *Neo*, neomycin resistance; *TK*, herpes simplex thymidine kinase promoter; *TF*, mouse tissue factor. (*b*) Luc expression system. SV_{40}, SV_{40} promoter; *Er*, SV_{40} enhancer; *TF-P*, tissue factor promoter; Amp^r, β-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 μl cold buffer C (20 mM Hepes-KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 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°C. For organs, the above protocol was modified according to Deryckere and Gannon (58). Pieces of tissue, 0.1 × 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 $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF, 0.6% NP-40). The tissue was homogenized in an Ultrathurrax (Wheaton Scientific, Milleville, 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 μl buffer B (25% glycerol, 20 mM Hepes-KOH, pH 7.9, at 4°C, 420 mM NaCl, 1.5 mM $MgCl_2$, 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°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: NFκB consensus: 5′-AGTTGAGGGGACTTTCCAGGC-3′, tissue factor–NFκB: 5′-AGGGTCCCGGAGTTTCCTACCGGGA-3′. EMSA were performed as described previously (30). Oligonucleotides were labeled to a specific activity $> 5 \times 10^7$ cpm/μg DNA using T4-polynucleotide kinase (Promega). Binding of NFκB 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 μg/μl poly dI/dC in a total of 20 μl as described essentially by Pahl et al. (60). 1 ng of labeled oligonucleotides (∼ 50,000 cpm Cerenkov) was added to 10 μg 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× TBE. The gels were run at room temperature with 30 mA for ∼ 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°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 μg of plasmid DNA (pGL_2-Luc, control plasmid pGL_2-basic, murine tissue factor expression plasmids, pXT1-control vector, IκBα or mutated Jun expression plasmids) was mixed with 150 μg 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 ml.

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_3PO_4; 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_2. Cryostat sections of mouse kidneys were fixed in acetone for 10 min and then incubated for 5 min in 0.6% H_2O_2 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_2O_2 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_2O_2 (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_3 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

	% binding activity (day 1 = 100%)								
Patient No.	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.

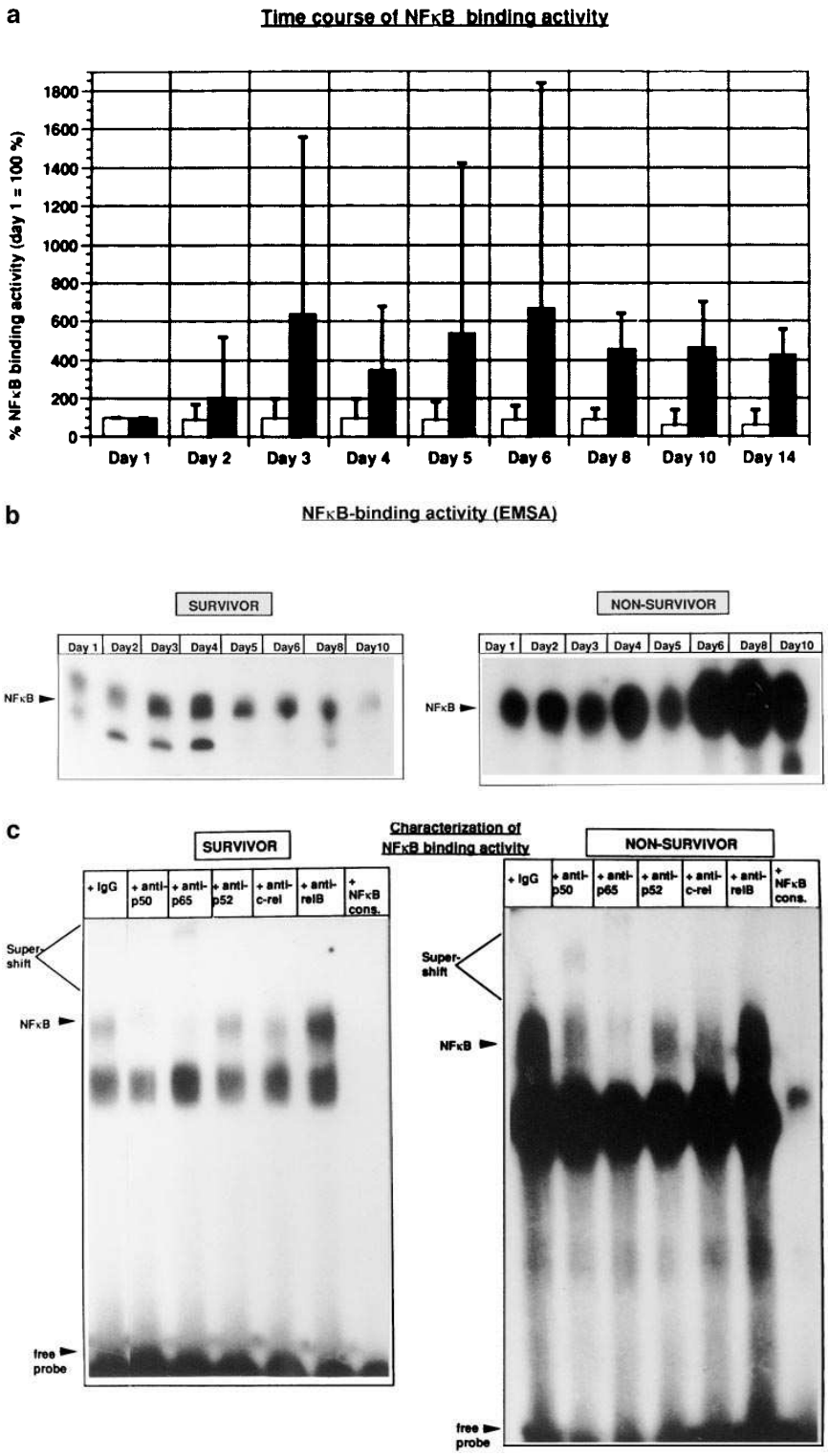

Figure 2. Time course of NFκB binding activity in PBMC of septic patients. (*a*) Time course of NFκB binding activity determined in 10 μg nuclear extracts of PBMC. Each EMSA signal obtained at the time points indicated (Tables I and II) was quantitated by laser densitometry. Signal intensity was determined at each day for all patients surviving ($n = 10$) or not surviving ($n = 5$) and the mean±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. 2 *c*).

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 analysis (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.

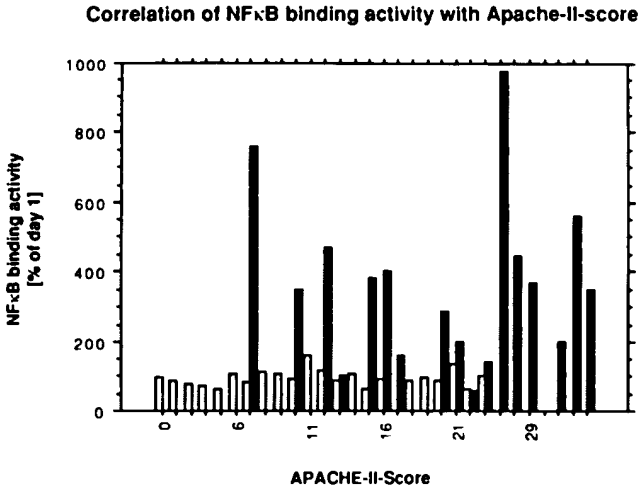

Figure 3. Correlation of NFκB binding activity with APACHE-II score. NFκB binding activity was determined with EMSA and quantitated by laser densitometry. The value obtained on day 1 was defined as 100%. The daily values obtained from the 10 patients surviving (*open bars*) and 5 patients not surviving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

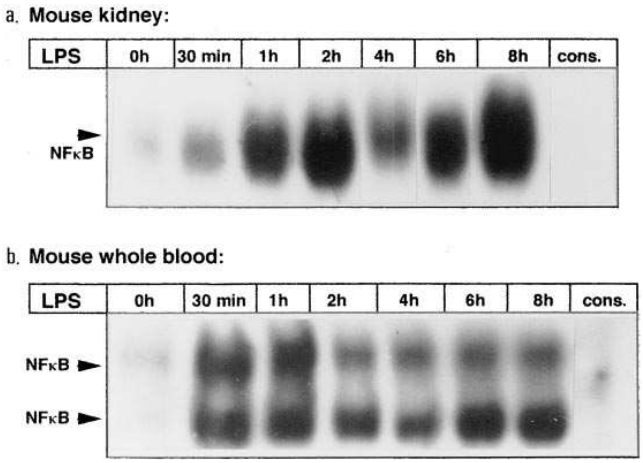

Figure 4. Time course of NFκB activation in kidneys and PBMC of LPS-treated mice. (*a*) Nuclear extracts were prepared from kidneys of mice that had received LPS (1.75 μg LPS and 15 mg D-galactosamine) for the times indicated. 10 μg of each nuclear extract was analyzed by EMSA for the DNA binding activity 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.*).

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_{40}-promoter driven Luc plasmid pGL_2-Luc and the promoterless control vector pGL_2-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_2-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_2-basic (Fig. 5 *b*, *right*, and Table IV). A similar transfection efficiency was found in isolated PBMC (Fig. 5 *c* and Table IV).

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

		% of correctly classified cases		
	Discriminant score	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.

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.

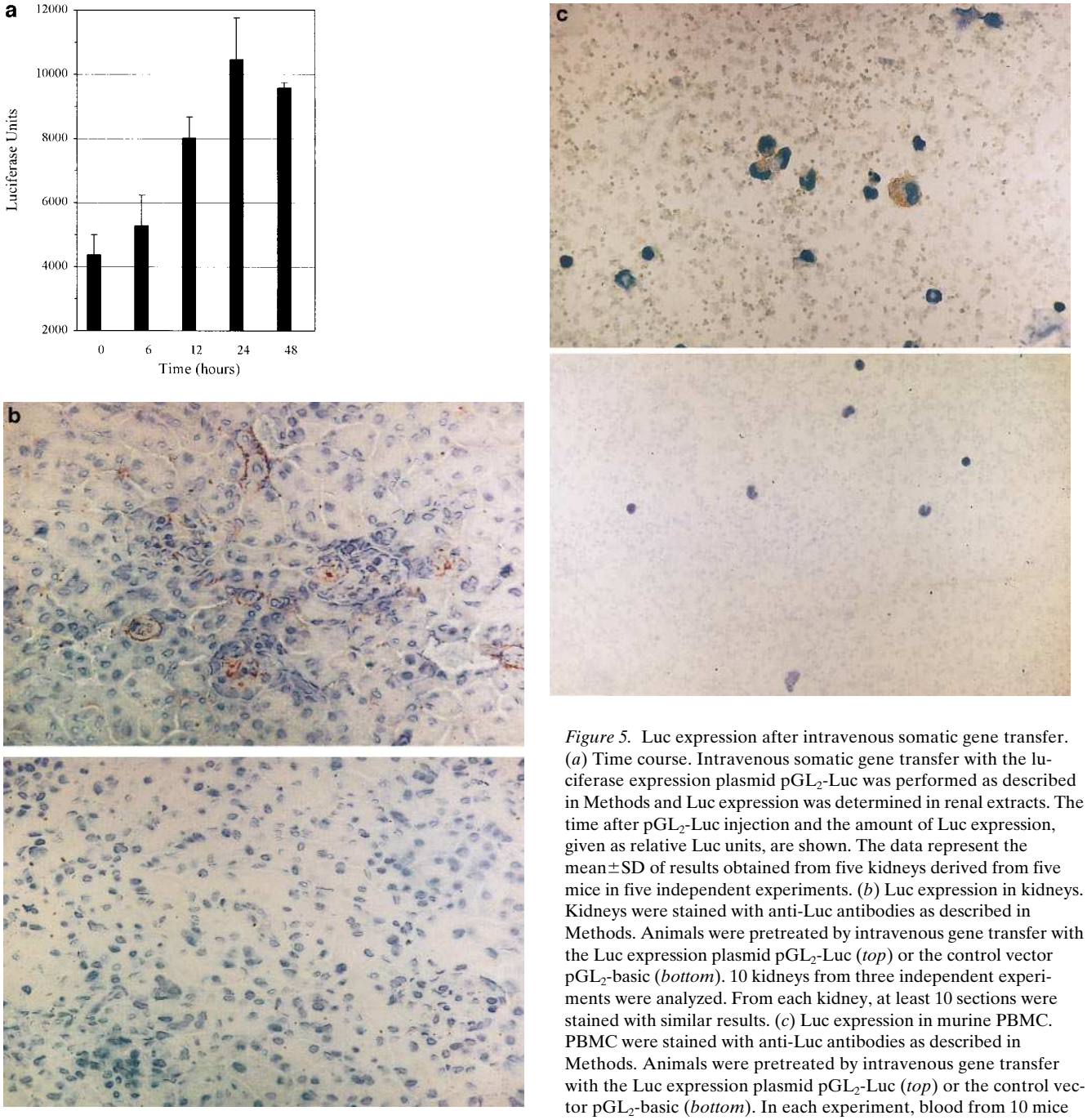

Figure 5. Luc expression after intravenous somatic gene transfer. (*a*) Time course. Intravenous somatic gene transfer with the luciferase expression plasmid pGL_2-Luc was performed as described in Methods and Luc expression was determined in renal extracts. The time after pGL_2-Luc injection and the amount of Luc expression, given as relative Luc units, are shown. The data represent the mean±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_2-Luc (*top*) or the control vector pGL_2-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_2-Luc (*top*) or the control vector pGL_2-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, IκBα was effective in suppressing LPS-mediated NFκB translocation to the nucleus (Fig. 7, *a* and *b*). When animals were pretreated with control vector pGL_2-basic alone, a strong induction of NFκB binding activity by LPS was observed in EMSA from renal extracts prepared as described in Methods. Pretreatment with IκBα (but not mutated jun) reduced the LPS-mediated NFκB activation. Similar data were obtained using the NFκB consensus motif (Fig. 7 *a*) or the NFκB binding region derived from the tissue factor promoter (Fig. 7 *b*).

Tissue factor, the central activator of the coagulation mechanism, is known to be involved in the lethal effects of LPS. Tissue factor transcription in vitro and in vivo is under control of NFκB and AP-1 (12–15, 30–34). Treatment of mice with LPS resulted in induction of renal NFκB binding activity to the tissue factor NFκB motif (Fig. 7 *b*) and induction of renal tissue factor antigen expression (Fig. 8, *A* and *B*). When intravenous somatic gene transfer with IκBα 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, IκB pretreatment reduced NFκB binding activity to the NFκB 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_2-Luc expression vector transfected)	% positive cells (pGL_2-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_2-Luc expression plasmid or the promotorless vector pGL_2-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).

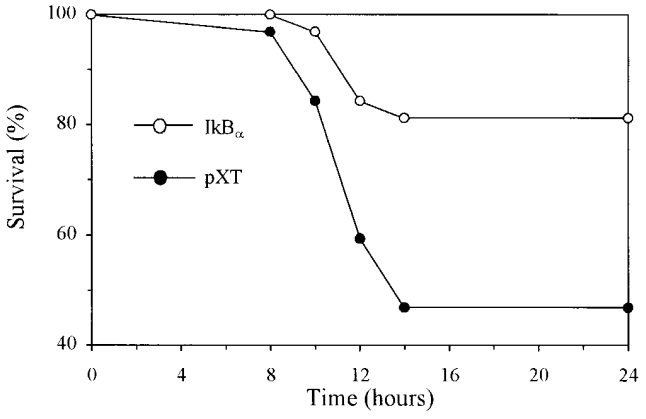

Figure 6. Effect of gene transfer with IκBα on LPS-mediated mortality. Survival was determined in mice treated with 1.75 μg LPS and 15 mg D-galactosamine (see Methods). 5 d and 24 h before LPS injection mice were treated with the IκBα expression plasmid ($n = 32$) or control vector pXT1 ($n = 32$) by 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 IκBα-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 IκBα 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 $pGLTF_4$ (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-

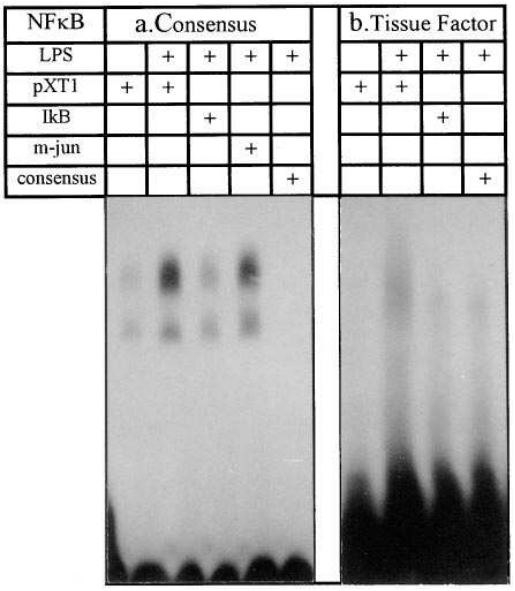

Figure 7. Effect of gene transfer with IκBα on LPS-mediated NFκB activation. Nuclear extracts from kidneys were prepared 8 h after injection of 1.75 μg LPS and 15 mg D-galactosamine. 10 μg of nuclear extract was incubated with the NFκB consensus sequence (*a*) or the NFκB motif derived from the tissue factor promoter (*b*). Shown is the effect of somatic gene transfer with either the control vector pXT1, IκBα, 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.

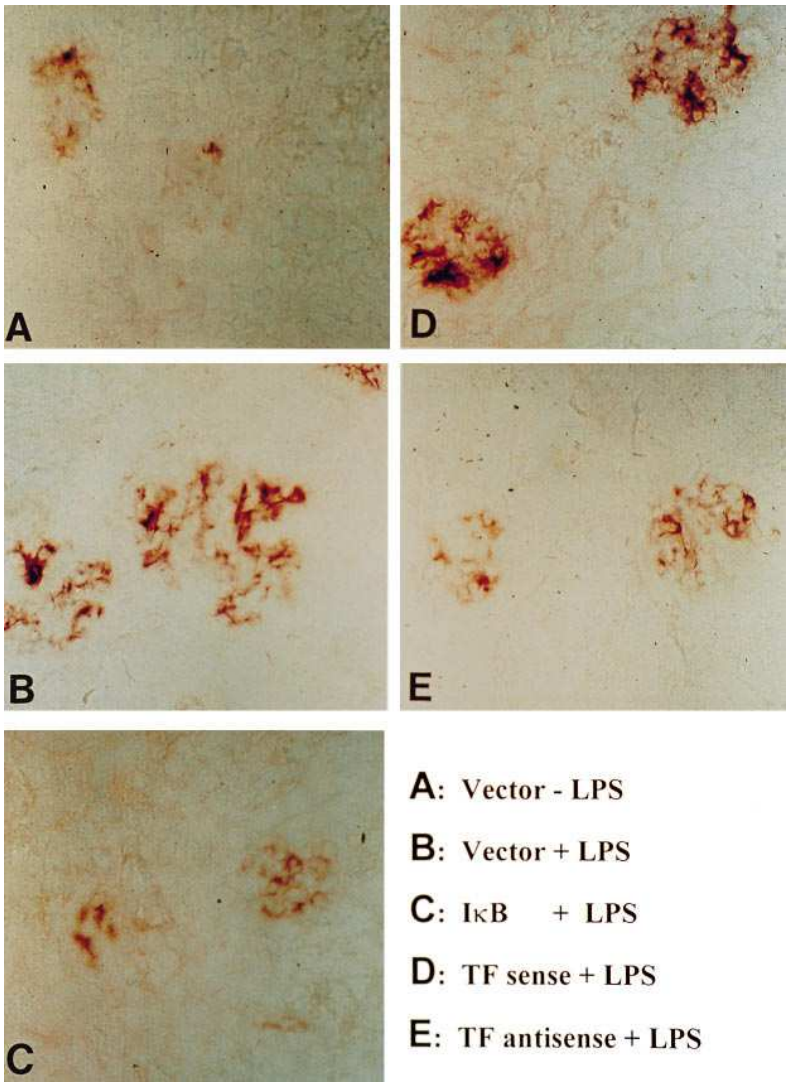

Figure 8. Effect of gene transfer with IκBα and tissue factor expression plasmids on the LPS-mediated tissue factor antigen expression. Kidneys were harvested 8 h after treatment with LPS (1.75 μg LPS, 15 mg D-galactosamine) and stained with an anti–tissue factor antibody. Animals were pretreated by 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 $pGLTF_4$ was dependent on NFκB. Mice were pretreated with the control vector pXT1 or IκBα together with $pGLTF_4$. 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

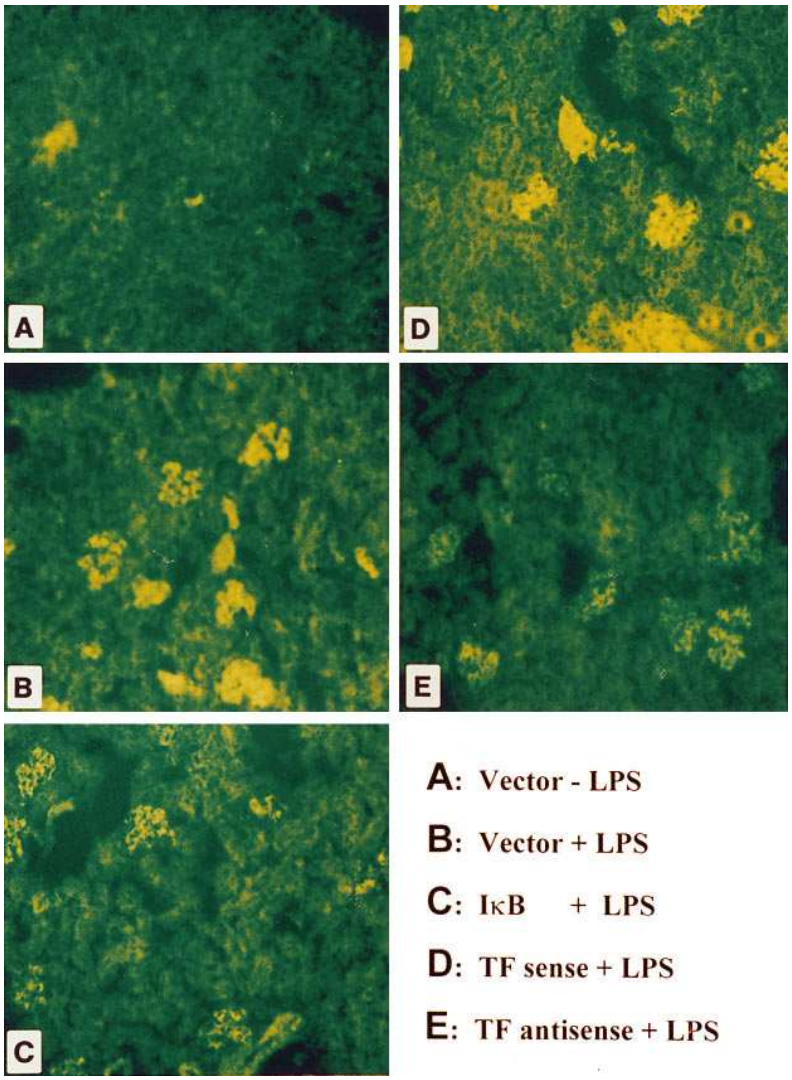

Figure 9. Effect of gene transfer with IκBα and tissue factor expression plasmids on LPS-mediated renal fibrin/fibrinogen deposition. Kidneys were harvested 8 h after treatment with LPS (1.75 μg LPS, 15 mg D-galactosamine) and stained with an anti–fibrin/fibrinogen antibody. Animals were pretreated by 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.

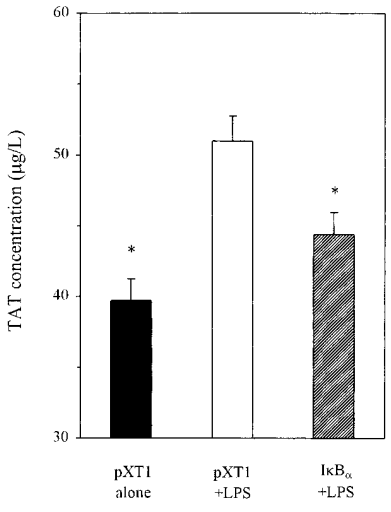

Figure 10. Effect of gene transfer with IκBα on LPS-mediated increase in TAT formation. TAT were determined in plasma of mice 8 h after treatment with LPS (1.75 μg LPS and 15 mg D-galactosamine). Mice were pretreated by intravenous somatic gene transfer with the control vector pXT1 alone (*black bar*,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

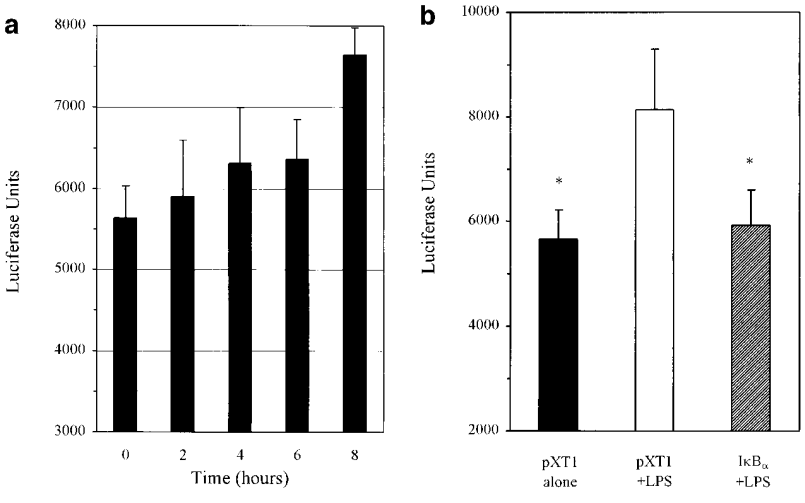

Figure 11. Time course of tissue factor promoter activity and the effect of gene transfer with IκBα on the tissue factor promoter activity. (*a*) Time course. Mice were treated by intravenous somatic gene transfer with the tissue factor promoter-Luc expression plasmid pGLTF4 (Fig. 1). 24 h after the second gene transfer, 1.75 μg 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±SD of 10 kidneys for each time point, given as relative Luc units. (*b*) Gene transfer with IκBα. Mice were pretreated by intravenous somatic gene transfer with control vector pXT1 (*black bar*, $n = 5$), pXT1 plus LPS (*open bar*, $n = 5$), or IκBα 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 administration of LPS (1.75 μg LPS and 15 mg D-galactosamine). The data shown represent the mean of the Luc determination performed in 10 kidneys±SD and are expressed as relative Luc units. 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.

tosamine conditioned the mice to mortality 8–12 h after LPS administration. This model allowed us to clearly define the role of NFκB 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 IκBα expression plasmid reduced LPS-mediated NFκB activation in renal extracts and increased survival after LPS. The experiments provide evidence that NFκB 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 (∼ 20–40%, Table IV). IκBα preferentially inhibits NFκB p50 and p65. The characterization of the proteins binding to the NFκB consensus motif also revealed the presence of other members of the NFκB family (Fig. 2 *c*). Thus, a complete inhibition of NFκB cannot be expected by gene transfer with IκBα. However, intravenous somatic gene transfer was at least partially effective as evidenced by the inhibition of NFκB 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 NFκB activation observed in patients has a predictive value in determining the outcome of sepsis.

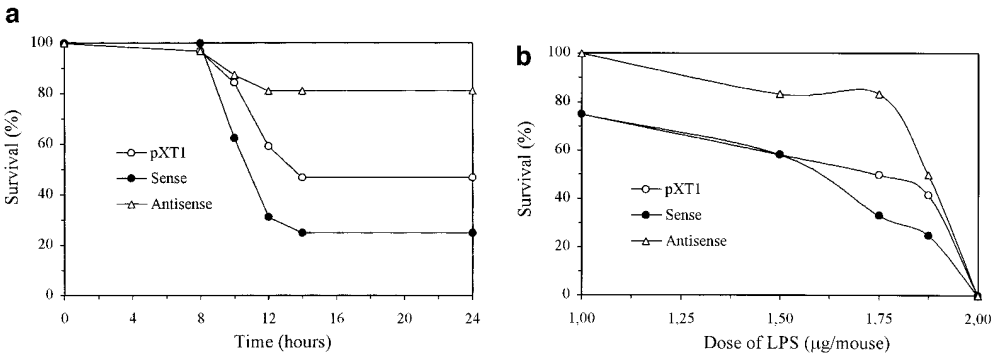

Figure 12. Effect of gene transfer with tissue factor expression plasmids on survival time. (*a*) The survival of mice treated with LPS (1.75 μg LPS and 15 mg D-galactosamine) was determined. Mice were treated with the tissue factor expression plasmids (*Sense*, $n = 32$; *Antisense*, $n = 32$; or *pXT1*, $n = 32$) 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 μg per mouse.

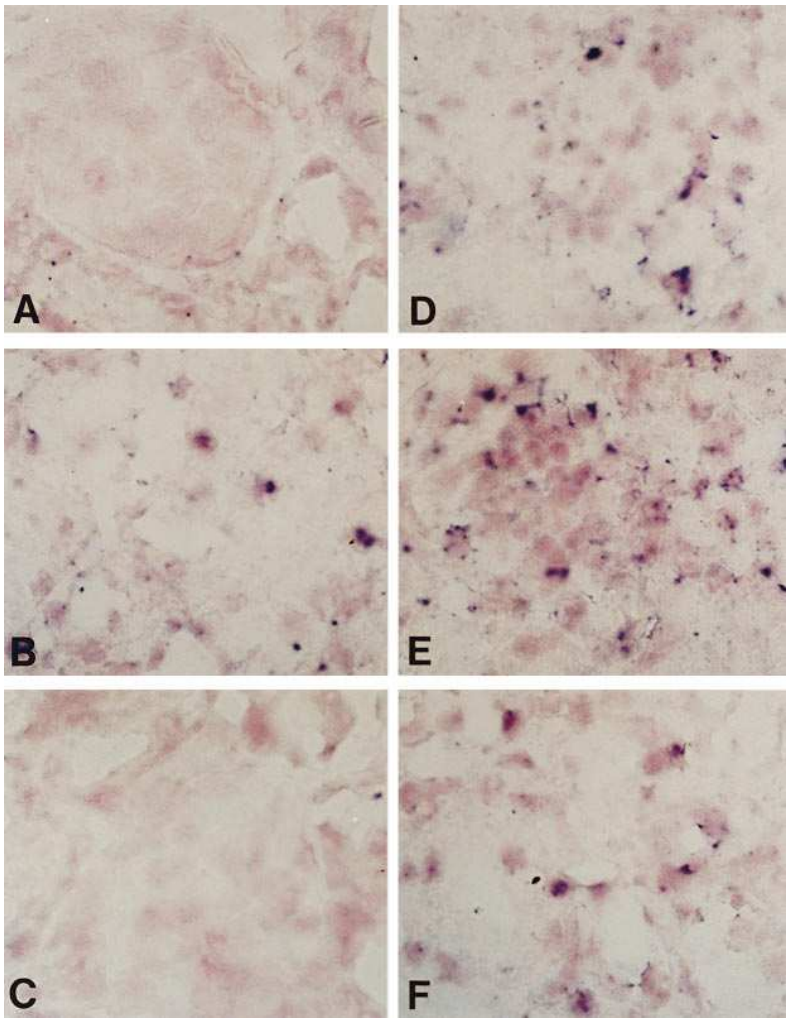

Figure 13. Effect of tissue factor expression plasmids on LPS-mediated renal tissue factor transcription. Kidneys were harvested 8 h after treatment with LPS (1.75 μg LPS, 15 mg D-galactosamine) and analyzed by in situ hybridization for the presence of tissue factor transcription. Animals were pretreated 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 IκBα was not effective when given simultaneously with or after LPS. Thus, IκBα has to be expressed before LPS is injected, which is compatible with NFκB-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 TNFα neutralizing antibodies in clinical trials. If this hypothesis is correct, then one has to assume that NFκB 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 TNFα 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, TNFα 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 TNFα neutralizing antibodies are critical for efficient reduction of endotoxemia-dependent mortality in animals (66), different timing of TNFα neutralizing antibodies might account for the conflicting results obtained in animal models and clinical trials. Cytokines other than TNFα might also contribute to septicemia-mediated NFκB activation and subsequent gene expression. Cytokine activation of NFκB lasts in vitro for only short time; therefore, we hypothesize that yet unknown mechanisms perpetuate NFκB activation and NFκB-dependent gene expression observed in prolonged inflammatory response. In contrast to the short-lasting NFκB activation induced by TNFα, we observed recently that ligands of the TNFα-inducible cellular receptor RAGE mediate perpetuated NFκB activation (Bierhaus, A., and P.P. Nawroth, unpublished observations). Thus, blocking TNFα by neutralizing antibodies might only in part inhibit LPS-dependent NFκB activation and therefore fail to inhibit pathways of NFκB activation downstream of TNFα. Furthermore, it has been reported that TNFα antibodies specifically inhibited fibrinolysis and thereby enhanced the risk for microvascular thrombosis (23). Thus, in the course of human sepsis side effects of TNFα 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-

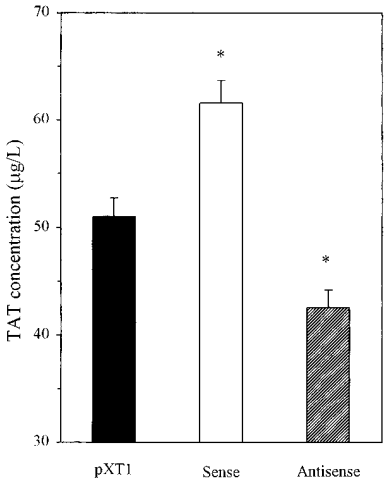

Figure 14. Effect of tissue factor on LPS-induced TAT formation. TAT were determined in mice 8 h after treatment with 1.75 μg LPS and 15 mg D-galactosamine. Mice were pretreated by intravenous somatic gene transfer with control vector pXT1 (*black bar*, $n = 5$), tissue factor sense (pXTF-s; *open bar*, $n = 5$), or tissue factor antisense (pXTF-as; *hatched bar*, $n = 5$). The data represent the mean of three independent experiments±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 TNFα-induced fibrin formation in tumor capillaries is in part dependent on NFκB-mediated tissue factor induction (30, 44). Therefore, tissue factor might well be an important target of LPS-induced NFκB activation in sepsis and endotoxemia. The data shown suggest that not only mobilization of cryptic tissue factor, but also de novo NFκB-dependent transcription of tissue factor mediate TAT formation and fibrin deposition that in part contribute to the lethal effects of LPS. Thus, NFκB activation plays an important role in tissue factor–mediated "consumption coagulopathy" (67). IκBα 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 TNFα-mediated fibrin deposition in capillaries of Meth-A-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 NFκB, 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 NFκB plays a central role in human disease and in an animal model of endotoxemia. Intravenous somatic gene transfer with IκBα can be used to analyze the contribution of NFκB to endotoxemia. Further applications of this approach may include ischemia–reperfusion and transplant rejection.

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