

Adaptation to Bacterial Lipopolysaccharide Controls Lipopolysaccharide-induced Tumor Necrosis Factor Production in Rabbit Macrophages

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Abstract

These experiments provide an explanation for the observation that two intravenous injections of lipopolysaccharide (LPS) spaced 5 h apart in rabbits cause tumor necrosis factor/cachectin (TNF) levels to rise in the blood only after the first LPS injection. Herein we show that treatment of elicited peritoneal exudate rabbit macrophages (PEM) with two doses of LPS given 9 h apart results in a marked reduction in TNF production by the second LPS exposure. This state of hyporesponsiveness is a result of adaptation to LPS, is induced by LPS concentrations that are 1,000-fold less than required to induce TNF production (picograms vs. nanograms), is characterized by a decrease in LPS-induced TNF mRNA without any change in TNF mRNA half-life, is not changed by including indomethacin in cultures, and is specific for LPS since LPS-adapted cells display a TNF response to heat-killed *Staphylococcus aureus* that is at least as good as that observed in control PEM. (*J. Clin. Invest.* 1990. 85:1108–1118.) endotoxemia • monokines • septicemia • shock • tolerance

Introduction

The importance of the cytokine tumor necrosis factor- α /cachectin (TNF)¹ as a mediator of host defense and inflammatory responses is now well documented (1). A key role for TNF has been established in models of shock induced by endotoxin (lipopolysaccharide, LPS) and gram-negative bacteria (1–3). LPS is a potent inducer of TNF's being active in the picomolar range (3). However, induction of TNF by LPS is under tight control as documented in a recent study from our laboratory where we showed that sequential intravenous injections of LPS results in TNF release into blood after only the initial LPS injection and not with the second LPS dose (3). The mechanism responsible for acquired hyporesponsiveness had not been defined although it could not be induced by infusion of

recombinant TNF alone eliminating mechanisms involving negative feedback by TNF or TNF-inducible cell products (3).

The diminished TNF response in vivo is most likely a result of decreased response to LPS by cells of the monocyte/macrophage lineage, the cell type that is the major source of LPS-induced TNF in vivo (1). This change in sensitivity to LPS might be explained by a process known as adaptation (4).

Tolerance or acquired hyporesponsiveness to the toxic effects of LPS has been recognized for many years although the mechanisms that control this are not well understood (reviewed in Johnston and Greisman [5]). Two distinct phases of endotoxin tolerance have been recognized: early-phase tolerance that is a lipid A-dependent phenomenon and protects against challenge with any LPS and late-phase tolerance that has O-antigen specificity and is dependent upon specific anti-LPS antibodies in the circulation. It is our hypothesis that adaptation to LPS at the level of the macrophage is an important feature of early-phase tolerance to the toxic effects of LPS. In this regard a recent report from this laboratory using the murine macrophage-like cell line RAW 264.7 cells establishes that LPS induces hyporesponsiveness to its own effects (6).

Studies described here with rabbits and with elicited rabbit peritoneal exudate macrophages (PEM) investigate the mechanism of acquired hyporesponsiveness to LPS measuring TNF production at the mRNA and protein level. We show that hyporesponsiveness to LPS is induced 6–9 h after exposure to trace amounts of LPS and is characterized by specific adaptation to LPS. Compared with control cells, LPS-adapted PEM require up to 1,000-fold more LPS to induce TNF although LPS-adapted PEM, in contrast, display dose-response curves for *Staphylococcus aureus*-induced TNF production that are very similar to control (nonadapted) PEM.

Methods

Animals. Outbred New Zealand white rabbits (male, 1.8–2.2 kg) were obtained from White's Rabbit Ranch, Vista, CA and maintained on a standard pelleted diet for 7–14 d before use.

In vivo studies. LPS-induced hyporesponsiveness was studied in New Zealand white rabbits which were fasted overnight and cannulated (femoral artery) under local anesthesia using 1% procaine as described previously (3). The rabbits were placed in restraining suits, which permitted the animals to stand or recline in the prone position without disrupting experimental procedures, and injections of LPS in sterile saline were made through the marginal ear vein. Blood samples collected using the femoral artery catheter were clotted in sterile glass tubes, and serum was stored at -20°C pending TNF assay.

Macrophage activators and inhibitors. *Salmonella minnesota* Re595 LPS was extracted from late log-phase growth cultures using the phenol chloroform petroleum ether method as described previously (3, 7). An additional rough strain (Re) LPS, *Escherichia coli* K12 D31m4 and the following smooth strain LPS, *E. coli* 0111:B4, *S. minnesota* (wild type) and *Salmonella typhimurium* were obtained from List Biological Laboratories, Inc., Campbell, CA. Stock solutions of LPS were prepared by suspending 10 mg of LPS in 2 ml of 20 mM EDTA and

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1. **Abbreviations used in this paper:** DEP, diethyl pyrocarbonate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PEM, elicited peritoneal exudate macrophages; R-, rough-form LPS; S-, smooth-form LPS; TNF, tumor necrosis factor/cachectin.

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sonicating until clarified (3–5 20-s bursts at maximum intensity using a W375 sonicator with a No. 419 microtip (Heat Systems-Ultrasonics, Farmingdale, NY). Aliquots of the LPS stocks (200 μ l) were stored at -20°C , and when thawed for use were sonicated for 15 s using a microsonicator (Kontes Co., Vineland, NJ). LPS working dilutions were prepared in 10 mM Hepes saline which was formulated using 1 M Hepes stock (Gibco Laboratories, Grand Island, NY) and sterile, non-pyrogenic saline (0.9% sodium chloride irrigation, USP, Travenol Laboratories, Deerfield, IL). Serial dilutions were made using sterile polypropylene pipettes and tubes, and each solution was vigorously vortexed for 15 s before transfer. Heat-killed *S. aureus*, a gift from Dr. Theo N. Kirkland, Veterans Administration Hospital, San Diego, CA, was a clinical isolate that was grown overnight in trypticase soy broth, washed in saline, and boiled for 2 h. The final stock concentration was 1×10^{11} cells/ml.

A 10 mM stock of indomethacin was prepared in 95% ethanol and diluted in serum-free RPMI 1640 immediately before use. Actinomycin D (1 mg/ml stock solution) was prepared in 10 mM Hepes saline.

Establishment of LPS-adapted peritoneal exudate macrophages. Media and solutions used for macrophage culture were prepared using sterile, tissue culture-grade plasticware. Glassware employed in media preparation was acid-cleaned and baked overnight at 200°C to inactivate endotoxin. RPMI 1640 was obtained as powered cell culture medium (Gibco Laboratories) and dissolved in sterile nonpyrogenic water (Travenol Laboratories) and supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, and 10 mM Hepes as described previously (3).

PEM were obtained by lavage 3 d after intraperitoneal injection of mineral oil in rabbits as described previously (3). The PEM were washed and cultured in serum-free RPMI 1640 in six-well clusters (3.5-cm wells, 5×10^6 PEM per well, 3 ml of medium) or 150-cm² flasks (8×10^7 PEM per flask, 40 ml of serum-free medium) followed by washing to remove nonadherent cells and replenishment of serum-free medium (1.25 ml per well, 20 ml per flask). In studies of adaptive responses, freshly prepared adherent PEM were exposed to primary doses (0.001–3,000 pg of LPS/ml) for 3–18 h followed by collection of conditioned medium, washing, replenishment of medium, and stimulation with a challenge dose of LPS (1–100 ng/ml). The conditioned medium was harvested 0–12 h after challenge, and cell viability was determined by phase-contrast fluorescence microscopy of cultures which had been incubated 15 m at 37°C , 5% CO with serum-free medium containing 1 $\mu\text{g}/\text{ml}$ fluorescein diacetate (8). In some cases the PEM were lysed by addition of guanidinium thiocyanate for preparation of total cellular RNA.

Cytolytic assay for TNF. TNF was measured using a cytolytic assay with actinomycin D (1 $\mu\text{g}/\text{ml}$)–treated L929 cells as described previously (3, 9). Each plate included TNF standard (conditioned medium from LPS-treated RAW 264.7 cells, 5×10^4 U/ml), which was calibrated using human recombinant TNF standard obtained from the National Institute for Biological Standards and Control, Hertfordshire, England. The coefficient of variation (SD/mean) for the assay was 0.1–0.15. Experiments with PEM were performed and assayed in duplicate; thus the experimental results represent the mean of four data points.

Prostaglandin assay. Prostaglandin E₂ levels in PEM-conditioned medium were measured using a radioimmunoassay as previously described (10).

Reagents and stock solutions for RNA preparation. All reagents used were analytical reagent grade. Cesium chloride and redistilled, crystalline phenol were obtained from Bethesda Research Laboratories, Gaithersburg, MD. Guanidinium thiocyanate and diethylpyrocarbonate (DEP) were obtained from Fluka BioChemika, Ronkonkoma, NY. Chelex 100 (200–400 mesh, sodium form) was obtained from Bio-Rad Laboratories, Richmond, CA. All glassware was acid-cleaned and heated at 180°C for 18 h before use. DEP-treated water was prepared by adding DEP (0.1% final concentration) to sterile distilled water, shaking vigorously, standing at room temperature overnight, and autoclaving at 121°C for 60 min. The following stock solu-

tions were prepared, chelex-adsorbed, DEP-treated, and autoclaved as described above: 4 M sodium chloride; 3 M sodium acetate, pH 6; 0.5 M EDTA, pH 8; 1 M sodium phosphate buffer, pH 7; 0.5 M Pipes, pH 6.8. The following solutions were prepared in sterile distilled water followed by chelex treatment and autoclaving (121°C , 20 min): 2 M Tris, pH 7.5; 1 M Tris, pH 8.

Tris-EDTA stock (20 \times) contained 10 ml of 2 M Tris, pH 7.5, and 4 ml of 0.5 M EDTA, pH 8, brought to 100 ml with DEP-water. Cesium chloride (5.7 M stock in 1 \times Tris-EDTA) was prepared using DEP-treated water, filtered (0.45 μm), and autoclaved. SDS (10% stock in sterile distilled water) was chelex-treated, followed by addition of DEP (0.1% final) and heating overnight at 60°C . Sodium hydroxide (10 M stock) was prepared using sterile distilled water. 20 \times SSC (3 M sodium chloride and 0.3 M trisodium citrate) was chelex- and DEP-treated and autoclaved as described above. Phenol was melted at 65°C , 8-hydroxyquinoline (0.1% final) was added, and three washes were performed using equal volumes of 1 M Tris, pH 8. The washed phenol was stored under nitrogen at 4°C and, when used for RNA extractions, was further equilibrated with sodium acetate buffer (0.01 M, pH 6) containing 0.1 M sodium chloride and 1 mM EDTA.

Lysis buffer (4 M guanidine thiocyanate, 25 mM trisodium citrate, 0.5% sodium lauroyl sarcosine, and 10 mM EDTA) was prepared using DEP-water, chelex resin (~ 100 mg/500 ml) was added with gentle stirring, and after standing for 60 min, the solution was filtered (11). Before use 2-mercaptoethanol and antifoam A were added to the lysis buffer at 0.7% and 0.33% final concentrations, respectively.

RNA preparation and Northern blotting analysis. After removal of medium, adherent PEM in 150-cm² flasks were lysed by addition of 4 M guanidinium thiocyanate. DNA was sheared by passing the lysate through a 21-gauge needle, and the lysate was transferred to a 16 \times 76-mm polyallomer tube, underlaid with 2.5 ml of 5.7 M CsCl using a spinal needle, and centrifuged 12 h at 40,000 rpm in a model 50 Ti rotor (Beckman Instruments, Inc., Palo Alto, CA) (12). The supernatant was aspirated and discarded and the RNA pellet was solubilized in 400 μ l of 1 \times TE and extracted twice with 500 μ l of phenol-chloroform (1:1) and once with 1 ml chloroform-isopentyl alcohol (95:5) followed by precipitation, wash, and reprecipitation in 70% ethanol in the presence of 0.3 M sodium acetate.

RNA was denatured using 1 M glyoxal and 50% dimethyl sulfoxide in 10 mM phosphate, pH 7, for 1 h at 50°C (13). Loading buffer (2.5 μ l, containing 2.9 M sucrose, 2 mM EDTA, 20 mM Tris, and 0.3% bromophenol blue) was added per 20- μ l sample (15 μg of RNA), and electrophoresis was performed in 1.2% agarose for 18 h at 1 V/cm with recirculation of the 10 mM phosphate, pH 7, running buffer. The gel was stained for 15 min at room temperature in 0.01% acridine orange in 10 mM phosphate, pH 7, followed by several washes in phosphate buffer over 2 h and examination under ultraviolet (UV) illumination. The RNA was transferred to nylon membranes using 20 \times SSC overnight, and the membranes were then UV-treated for 2 min and heated at 80°C for 2 h to fix the RNA to the filters (14).

Probes for TNF and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were prepared from synthetic oligonucleotides synthesized by Research Genetics, Huntsville, AL, based on the published nucleotide sequence of rabbit TNF and GAPDH as shown below (15, 16):

TNF coding strand corresponding to amino acid residues 67–86:

5'-TGCCGCTCCTACGTGCTCCTCACTCACACT
GTCAGCCGCTTCGCCGTCTCCTACCCGAAC-3'

TNF antisense strand corresponding to amino acid residues 83–102:

5'-GGTCTCCCGGTGGCAGGGGCTCTTGATGGC
AGAGAGGAGGTTGACCTTGTTCCGGGTAGGA-3'

GAPDH coding strand corresponding to amino acid residues 43–62:

5'-ATGTTCCAGTATGATTCCACCCACGGCAAG
TTCCACGGCACGGTCAAGGCTGAGAACGGG-3'

GAPDH antisense strand corresponding to amino acid residues 59–78:

5'-ATCTCGCTCCTGGAAGATGGTGATGGCCTT
CCCCTTGATGACCAGCTTCCCGTTCTCAGC-3'

Probes radiolabeled with ^{32}P for use in Northern blot hybridizations were synthesized as follows. Coding strand and antisense strand (100 ng each in 0.5 μl) were pipetted into a 1.5-ml conical polypropylene tube containing 20 μl of water, boiled for 2 min, and cooled to room temperature for 10 min, and the following additions were made in a total volume of 60 μl : 150 μCi dATP (3,000 Ci/mmol) unlabeled dCTP, dGTP dTTP (25 μM final), Klenow buffer (7 mM sodium chloride, 7 mM Tris (pH 7.5), 7 mM MgCl₂, 5 mM dithiothreitol), Klenow fragment of DNA polymerase I (7 U, Promega Biotec, Madison, WI). After 1 h at room temperature, the probe was separated from the reaction mixture by phenol-chloroform extraction (using phenol equilibrated with 1 \times TE containing 100 mM sodium chloride and 0.02% SDS) and gel filtration.

Nylon membranes containing RNA samples were prehybridized 6 h in 60°C, pH 7, 50 mM Pipes, 100 mM NaCl, 50 mM sodium phosphate, 0.1 mM EDTA, 5% SDS containing 200 μg of yeast RNA and 50 μg of salmon sperm DNA per milliliter. The hybridization buffer was discarded and replenished with higher concentrations of RNA (750 $\mu\text{g}/\text{ml}$) and DNA (275 $\mu\text{g}/\text{ml}$), and freshly made ^{32}P oligonucleotide probe (1×10^7 cpm per filter), denatured using 50% formamide at 100°C immediately followed by equilibration on ice for 10 min, was diluted in buffer and added to the hybridization mixture. After 18 h at 60°C the hybridization buffer was discarded, and the filter was washed twice for 5 min in 1 \times SSC followed by one 15-min wash at 55°C. Autoradiography was performed using X-OMAT AR film (Eastman Kodak Co., Rochester, NY), which gave suitable exposures after 4–18 h. In some experiments the amount of ^{32}P probe hybridized on the Northern blots was quantitated using an Ambis Beta Scanning System (Automated Microbiology Systems, Inc., San Diego, CA).

Inclusion of a 0.24–9.5-kb RNA ladder (Bethesda Research Laboratories) in glyoxal gels and visualization of the bands with acridine orange staining under UV illumination indicated that TNF and GAPDH mRNA were present as single species migrating at 1.8 and 1.4 kb, respectively.

Results

In vivo induction of hyporesponsive state. Previously we reported that in rabbits injection of 10 μg of Re595 LPS produced a rapid, but transient increase in blood levels of TNF and that injection of the same dose of LPS 5 h later produced markedly reduced or no detectable intravascular TNF (3). Here we describe additional dose-response studies in rabbits injecting varying doses of Re595 LPS at $t = 0$ and 10 μg of LPS at $t = 300$ min. These are referred to as the primary and challenge LPS dose, respectively. The primary dose of LPS was varied between 0.02 and 10 μg of LPS/ml and blood samples were withdrawn for analysis of TNF levels in blood over an 8-h period. The results of this experiment shown in Fig. 1 indicate that a primary LPS dose of as little as 0.2 μg of LPS produces a marked reduction in TNF in blood induced by 10 μg of LPS administered 5 h later.

TNF production in PEM: induction of hyporesponsiveness. The first step towards determining the mechanisms at the cellular level that result in the hyporesponsive state is to establish the dose dependency for LPS-induced TNF production in elicited PEM comparing several different rough (R)- and smooth (S)-form isolates of LPS. (For definitions of R and S forms, see below.) Varying concentrations of LPS were added to PEM and after 12 h cell-free supernatants were removed and assayed for TNF. The results shown in Fig. 2, a (S-LPS) and b (R-LPS),

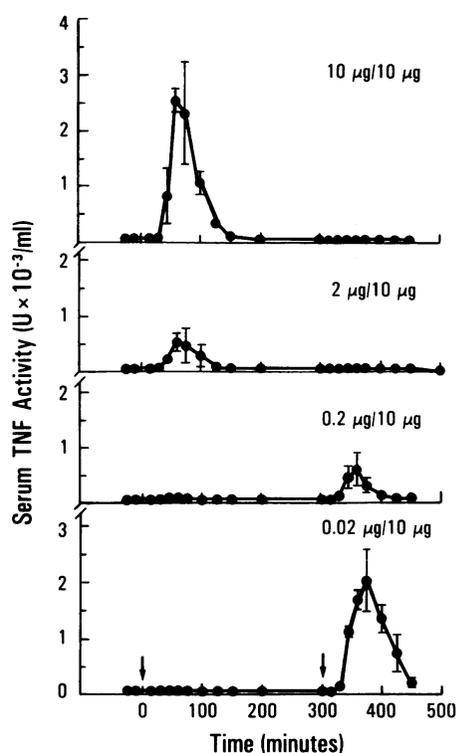


Figure 1. Induction of LPS hyporesponsiveness in rabbits. Femoral artery catheters were placed using local anesthesia, and the animals were maintained in restraining suits for the duration of the monitoring period. LPS injections were made through the marginal ear vein, and blood was collected using the femoral artery catheter. Primary injections of LPS (10, 2, 0.2, or 0.02 μg) were followed 300 min later by 10- μg LPS challenge. Mean \pm SE ($n = 4$).

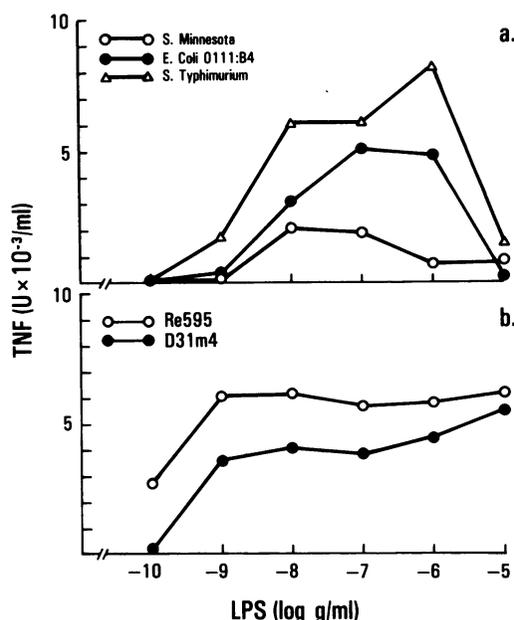


Figure 2. Induction of TNF by rough and smooth form LPS in rabbit PEM. PEM were cultured as described in Methods, and smooth form LPS (*E. coli* 0111:B4, *S. minnesota* wild type, or *S. typhimurium*) or rough form LPS (*S. minnesota* Re595 or *E. coli* K12 D31m4) were added to the cultures, and 18-h conditioned medium was harvested for TNF assay.

Table I. LPS-induced Adaptation to Its Own Effects: Time Dependency for Establishment of Adapted Cells

Primary LPS dose pg/ml	Exposure time to primary LPS dose h			
	3	6	9	18
0	91	101	92	70
100	102	32	17	18
300	107	22	19	13

See Methods for details of experimental protocol.

* TNF activity induced by challenge dose of 100 ng/ml for 12 h.

demonstrate that both forms of LPS induce TNF to about the same extent (5,000–10,000 U/ml), but that R-LPS is maximally active at concentrations 1–2 logs less than S-LPS. The apparent inhibition of TNF production by S-LPS concentrations > 1 µg/ml is not caused by decreased cell viability as determined by fluorescein diacetate staining of the cells. This inhibition is never observed with R-form LPS. In data not shown here, we established that TNF production by either R- or S-LPS is maximal 12 h after stimulation. For the remainder of the experiments described here, we used LPS from *S. minnesota* Re595 (R-form) and *E. coli* 0111:B4 (S-form).

To establish that LPS would induce a hyporesponsive state with PEM and to determine the time dependency, the following experiment was performed. PEM were cultured with 0, 0.1, or 0.3 ng/ml Re595 LPS for varying times up to 18 h, the primary LPS dose was removed by washing the cell monolayer, and the cells were exposed to a challenge dose of 100 ng/ml Re595 LPS for 12 h. Measurements of TNF in cell-free supernatants shown in Table I provide evidence that the LPS-induced hyporesponsive state occurs within 6 h and is maximally expressed after 9 h of exposure to the primary LPS dose.

Table II. TNF Production by the Primary LPS Dose

Primary LPS	[LPS]	[TNF]
	pg/ml	U/ml
Re595	—	<50
	0.01	<50
	0.3	<50
	10	<50
	100	400
	3000	12,000
0111:B4	—	<30
	0.001	<30
	0.1	<30
	10	<30
	1000	40

PEM were maintained for 9 h at 37°C, 5% CO₂ in the presence of the primary LPS dose and the aspirated medium was assayed for TNF with the L929 assay. Data of TNF production after addition of the challenge LPS dose are shown in Figs. 3 and 4.

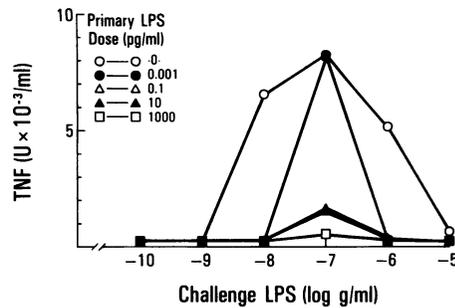


Figure 3. 0111:B4 LPS induction of hyporesponsiveness to LPS in PEM. Primary doses of 0111:B4 LPS (0–1,000 pg/ml) were added to PEM for 9 h, the conditioned medium was harvested and replenished, and the cells were restimulated with challenge doses of 0111:B4 LPS ranging from 0.1 ng to 10 µg/ml. Conditioned medium was harvested 12 h after challenge for TNF assay.

The next experiments compared different primary doses of Re595 and 0111:B4 LPS in establishing hyporesponsiveness and determined the effect of varying the challenge LPS dose. The results of this experiment are in Table II showing TNF production induced by the primary LPS dose and in Fig. 3 (0111:B4 LPS) and 4 (Re595 LPS) displaying LPS dose-response curves for TNF production after the LPS challenge dose. These data show that induction of hyporesponsiveness by the primary LPS dose occurs in the absence of detectable TNF, that both S- and R-LPS induce hyporesponsiveness, and that, depending upon the concentration of the primary LPS dose, hyporesponsiveness can be partially reversed by increasing the LPS concentration in the challenge dose. Thus the PEM response to the challenge dose of LPS has the characteristics of an adaptive change (4) and will be referred to as such in the remainder of this report.

The previous experiments utilized the same LPS in the primary and challenge treatments and demonstrated qualitatively similar results. We next sought to establish if R-form LPS could induce adaptation hyporesponsiveness to S-form LPS and vice versa. The PEM were exposed to the R- and S-form LPS in the primary dose and the alternative LPS form used for the challenge dose. The results of this experiment in

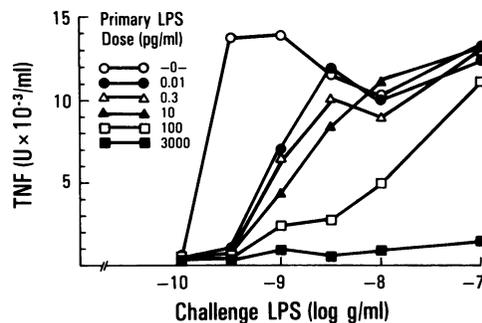


Figure 4. Re595 LPS induction of hyporesponsiveness to LPS in PEM. Primary doses of Re595 LPS (0–3,000 pg/ml) were added to PEM for 9 h, the conditioned medium was harvested and replenished, and the cells were restimulated with challenge doses of Re595 LPS ranging from 0.1 to 100 ng/ml. Conditioned medium was harvested 12 h after challenge for TNF assay.

Table III demonstrate that regardless of the form of the LPS in the primary or the challenge dose the PEM are adapted to LPS.

In experiments not shown we established that there are no inhibitors of the TNF bioassay released by the PEM at any time during the experiment and that nothing present in cell-free supernatants of adapted PEM inhibits TNF production by LPS when this supernatant is transferred to new cell cultures.

No TNF activity was detected in lysates of LPS-adapted cells and, when these lysates were mixed with a known amount of recombinant TNF, full activity of the added TNF was recovered. When PEM were biosynthetically labeled (100 μ Ci of [³⁵S]cysteine, 5×10^6 PEM, 1 ml of cysteine-deficient medium) immunoprecipitable ³⁵S-labeled TNF (migrating at 18 kD in SDS-PAGE) was readily detected in the conditioned medium of Re595 LPS-stimulated (1 ng/ml) cells. In contrast, [³⁵S]TNF was not detected in conditioned medium of PEM that had been exposed to 100 pg of Re595 LPS 9 h before challenge with 1 ng of LPS/ml. Control experiments also showed that conditioned medium of LPS-adapted macrophages did not inhibit immunoprecipitation or detection of [³⁵S]TNF.

Effect of indomethacin on LPS-induced adaptation. Prostaglandins have been shown to inhibit LPS-induced TNF production by macrophages (17). To evaluate the role of arachidonate-derived mediators in the induction of the hyporesponsiveness, we used the cyclooxygenase inhibitor, indomethacin, added to PEM together with the primary LPS dose and washed out of the cultures before the challenge dose of LPS. Data shown in Table IV demonstrate that indomethacin inhibits PGE₂ production > 95% but has no effect on adaptation of the PEM induced by the primary LPS dose. The TNF response to 100 ng/ml of Re595 LPS was increased approximately four-fold in the presence of 100 μ M indomethacin; this is consistent with other published reports demonstrating enhancement of LPS-induced monokine production by indomethacin (18, 19).

Investigation of possible mechanisms for LPS-induced adaptation to its own effects. Potential mechanisms to account for LPS-induction of hyporesponsiveness to its own effects include the induction of intracellular products that have a global effect on transcription or translation of the TNF gene, an increase in the rate of degradation of TNF mRNA, or a defect in the ability of cells treated with a primary LPS dose to

Table III. Crossed Adaptation of TNF Response Induced by Smooth and Rough Form LPS

		[LPS]		
		Primary	Challenge	
0111:B4	Re595	0111:B4	Re595	[TNF]
				U/ml
				ng/ml
—	—	100	—	6,600
—	3	100	—	470
—	—	—	1	5,900
3	—	—	1	1,600

PEM were maintained for 9 h at 37°C, 5% CO₂ in the presence or absence of LPS (primary dose) followed by washing and incubation for 12 h in the presence of LPS (challenge dose). TNF activity in the supernatants of the PEM was measured using the L929 assay.

Table IV. Effect of Indomethacin on LPS-induced Adaptation

[Indomethacin]	[Primary LPS dose]	[TNF]	[PGE ₂]
μ M	ng/ml	U/ml	nM
—	—	13,000	18
—	0.1	3,600	15
—	3	510	12
1	—	37,000	0.48
1	0.1	5,600	0.62
1	3	550	0.45

Indomethacin and primary Re595 LPS doses were added to PEM. After 9 h at 37°C, 5% CO₂, the cells were washed and challenged with 100 ng of LPS/ml for 12 h in the absence of indomethacin.

recognize LPS and properly initiate the signal transduction process. Experiments described below address some of these points.

It was first necessary to quantitate the LPS concentration dependence and kinetics of TNF mRNA production by PEM. Re595 LPS (0.1–100 ng/ml) was added to cells and at 0.5, 1.5, 4.5, and 8 h, supernatants were removed for TNF assay and total RNA was prepared for Northern blot analysis as described in Methods. The results of this experiment are shown in Figs. 5 and 6. To establish that comparable amounts of total RNA are present in each sample analyzed for TNF mRNA we also used a probe for GAPDH mRNA. These data show that the rate of TNF mRNA accumulation and the rate of TNF protein release is dependent upon the amount of LPS added. Typically we find no constitutive expression of TNF mRNA in cells before the addition of LPS. Comparable studies with 0111:B4 revealed similar findings except that as predicted from the LPS dose response of Fig. 2 a 0111:B4 LPS requires 10–100 higher concentrations than Re595 LPS to achieve similar TNF induction (data not shown).

The effect of adaptation on TNF mRNA induction and TNF release was next studied with 0111:B4 or Re595 LPS. Studies with Re595 LPS were performed as follows; macrophages were given a primary LPS dose of 0, 100 or 3,000 pg/ml for 9 h, the supernatant was removed, and the cells washed before addition of challenge LPS dose of 1 or 100 ng/ml. Samples of supernatant were removed for TNF assay and total RNA isolated from the cells at 1.5, 4.5, and 8 h after addition of the challenge LPS dose. The results of this experiment shown in Fig. 7 demonstrate that the primary LPS dose causes a marked reduction in the amount of TNF mRNA induced by a challenge with 1 ng/ml Re595 LPS. In contrast, challenge with 100 ng/ml Re595 LPS results in TNF mRNA induction and release of TNF. Results of measuring TNF in the PEM supernatant are shown in Fig. 8. A similar experiment was performed with 0111:B4 LPS except that the challenge LPS dose was 10 ng/ml; data from this experiment are shown in Figs. 9 (Northern blots) and 10 (TNF measurements).

Several different reports have provided evidence for post-transcriptional control of LPS-induced TNF mRNA expression (20–23). The reduction in steady state TNF mRNA accumulation observed in LPS-adapted cells could occur if the rate of degradation of TNF mRNA was increased. Therefore we measured the half-life of TNF mRNA using actinomycin D

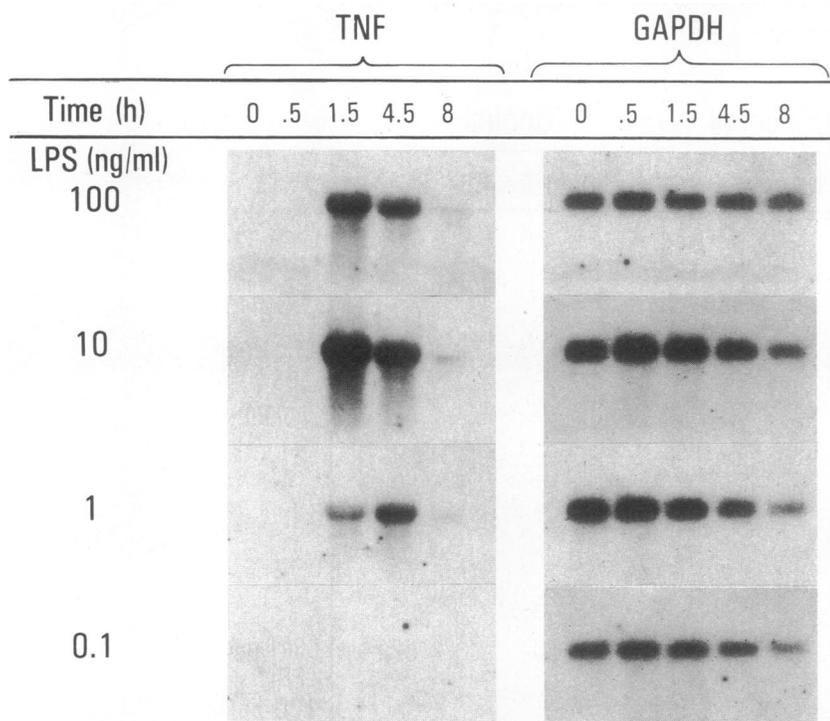


Figure 5. Kinetics of induction of TNF mRNA in Re595 LPS-stimulated PEM. Re595 LPS (0.1–100 ng/ml) was added to PEM, and at various times after stimulation, the conditioned medium was harvested, total cellular RNA was prepared, and Northern blot analysis was performed using ^{32}P -labeled rabbit TNF and GAPDH cDNA probes as described in Methods.

(5 $\mu\text{g}/\text{ml}$) to inhibit RNA synthesis. Cells were first treated with 0, 0.1, or 3 ng/ml Re595 LPS for 9 h, washed, and challenged with 100 ng/ml Re595 LPS for 2 h at which time actinomycin D was added; total RNA was prepared from cells 0, 0.5, 1, 2, 3, and 4 h after actinomycin D addition and the amount of TNF mRNA remaining was measured by Northern blot analysis as described in Methods. Northern blots were also quantitated by measuring ^{32}P probe hybridized on the filters as noted in Methods. The Northern blots are shown in Fig. 11 and the inset shows a semilog plot of the ^{32}P data obtained from scanning the blots. The calculated half-life for TNF mRNA for each experimental condition is in Table V, and these data show that adaptation does not result in a change in TNF mRNA half-life.

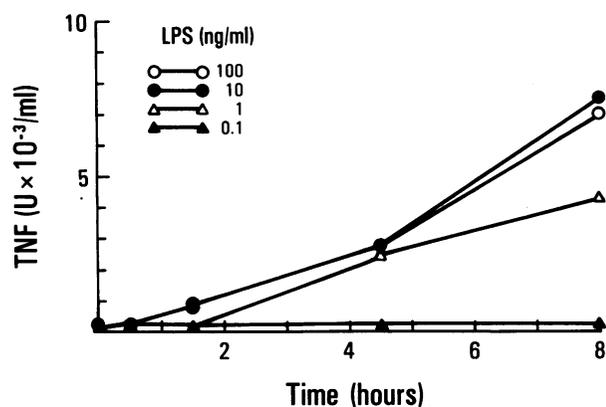


Figure 6. Kinetics of induction of TNF activity in Re595 LPS-stimulated PEM. Re595 LPS (0.1–100 ng/ml) was added to PEM, and conditioned medium was harvested at various times for 0–8 h after LPS stimulation for assay of TNF activity.

TNF induction by *S. aureus* in LPS-adapted cells. We next asked if the adaptation to LPS was specific for LPS or resulted from a global inhibition of TNF induction that is stimulus-independent. To address this question we used varying dilutions of a suspension of heat-killed *S. aureus* to induce TNF in cells treated with a primary LPS doses of Re595 LPS for 9 h. The results of this experiment are shown in Fig. 12 where *S. aureus* induced TNF production in LPS-adapted cells or control cells is compared. Here the primary LPS doses (0.01–100 $\mu\text{g}/\text{ml}$, Re595 LPS, see Fig. 4) that cause substantial desensitization to LPS produce a hyperresponsive state to *S. aureus* with as much as a threefold increase in TNF production up to levels of 3×10^4 U/ml. Even a primary LPS dose of 3 ng/ml results in only partial inhibition of *S. aureus*-induced TNF production despite having a marked effect on LPS-induced responses. Thus adaptation to LPS is specific for LPS.

Discussion

Previous studies from our laboratory (3) and data in this report show that in vivo LPS-induced TNF production is under tight control since a single, intravenous injection of LPS in rabbits is sufficient to render the animal hyporesponsive to a second LPS challenge administered several hours later. To determine if a similar phenomenon can be demonstrated at the level of the macrophage and to investigate the mechanism of the hyporesponsiveness we have used R- and S-form LPS to define conditions for making rabbit peritoneal exudate macrophages hyporesponsive to LPS and to further characterize LPS-induced TNF production by these cells. Herein we show that treatment with two doses of LPS given 9 h apart results in development of hyporesponsiveness, that S- and R-form LPS induce hyporesponsiveness at doses that are 1,000-fold less than required to induce TNF production (picograms LPS per

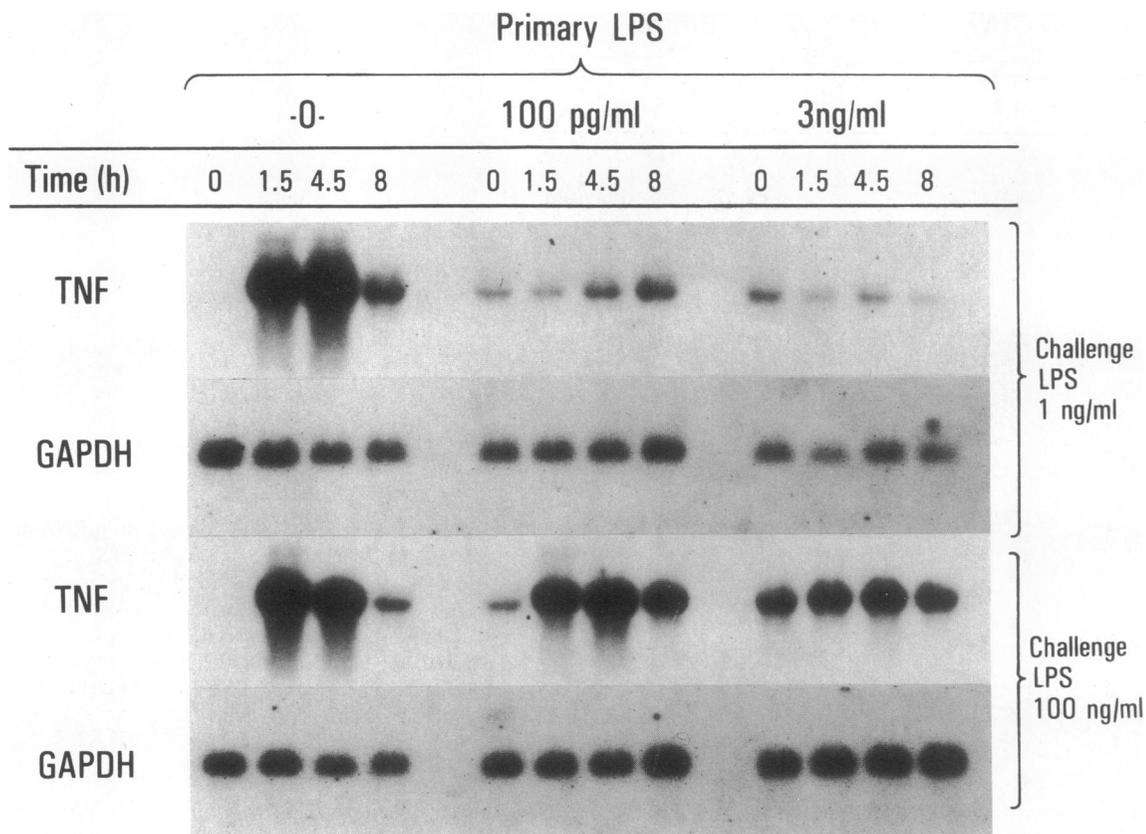


Figure 7. Kinetics of TNF mRNA response to Re595 LPS challenge (1 ng or 100 ng/ml) in control PEM or LPS-adapted PEM (exposure for 9 h to primary LPS doses of 100 or 3,000 pg/ml of Re595 LPS). Conditioned medium was harvested 0–8 h after challenge, total cellular RNA was prepared, and Northern blot analysis was performed using ³²P-labeled rabbit TNF and GAPDH cDNA probes as described in Methods.

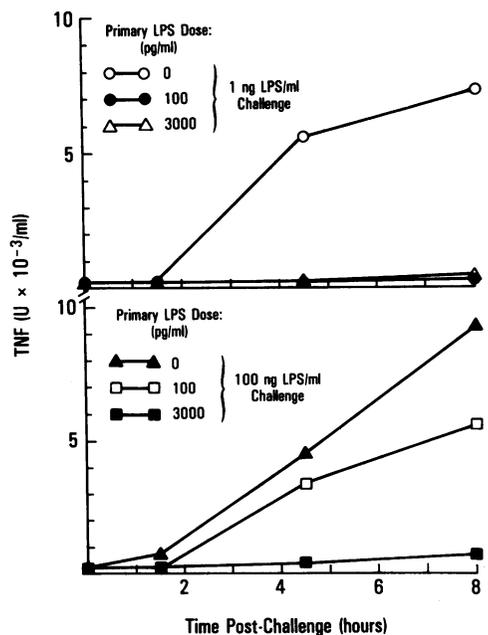


Figure 8. Kinetics of TNF response to Re595 LPS challenge (1 or 100 ng/ml) in control PEM or LPS-adapted PEM (exposure for 9 h to primary LPS doses of 100 or 3,000 pg/ml of Re595 LPS). Conditioned medium was harvested 0–8 h after addition of the challenge LPS dose for assay of TNF activity.

milliliter vs. nanograms LPS per milliliter), that hyporesponsiveness is a result of adaptation (4) to LPS, that adaptation is characterized by decreased accumulation of TNF mRNA without a change in the half-life of the TNF mRNA, that eicosanoids do not participate in induction of the hyporesponsive state, and that the LPS-adapted cells are stimulated by heat-killed *S. aureus*, to produce TNF levels that are equal to or greater than that of control cells.

LPS-induced TNF production by elicited peritoneal exudate macrophages. Elicited rabbit peritoneal exudate macrophages provide an excellent system to study LPS-induced TNF production since these cells can be obtained in large numbers and respond to both R- and S-form LPS preparations. S-form LPS isolates are maximally active at concentrations of 10–100 ng/ml, but are inhibitory between 1 and 10 μ g/ml (see Fig. 2 a). In contrast, R-form LPS isolates such as Re595 LPS maximally induce TNF at concentrations of 1 ng/ml and show no inhibition even when added at 10 μ g/ml. The mechanism of the inhibitory limb of the S-form LPS dose-response curve is unknown, although it is not a result of decreased cell viability.

We also describe the concentration dependence and kinetics of LPS-induced increases in steady-state levels of TNF mRNA using an oligonucleotide probe based on the cDNA sequence for rabbit TNF (15). Both the rate and extent of increase in steady-state levels of TNF mRNA are dependent on the amount of LPS added initially. These changes in TNF mRNA are qualitatively similar to changes reported by others

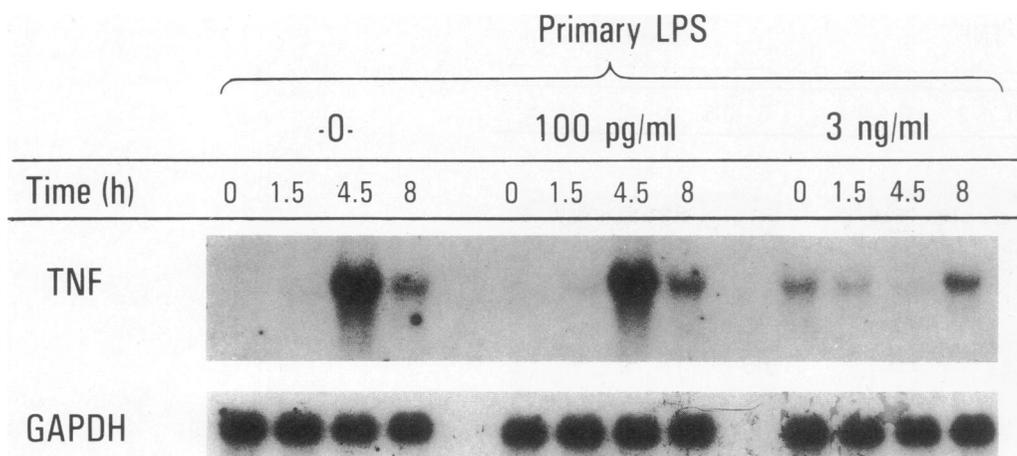


Figure 9. Kinetics of TNF mRNA response to 0111:B4 LPS challenge (10 ng/ml) in control PEM or PEM adapted by preexposure for 9 h to primary doses of 100 or 3,000 pg/ml of 0111:B4 LPS. Conditioned medium was harvested 0–8 h after challenge, total cellular RNA was prepared, and Northern blot analysis was performed using ³²P-labeled rabbit TNF and GAPDH cDNA probes as described in Methods.

using primary cultures of monocytes/macrophages stimulated with LPS (17, 20, 21, 24, 25). However, the concentrations of LPS used here to produce maximal stimulation of TNF are as much as 1,000-fold lower than used by other investigators, and in the case of Re595 LPS are in the same range that we used to maximally stimulate TNF production in vivo (3) and similar to doses reported to maximally induce TNF in whole blood ex vivo (26).

Characteristics of LPS-induced adaptation to its own effects. We established that the sensitivity of PEM to LPS measured by TNF production can be modulated by exposure of cells to LPS (primary dose) for 6–9 h before a second LPS exposure (challenge dose). Desensitization to LPS is induced by S- and R-form LPS with as little as 0.1 pg/ml of LPS being active in the primary dose. The biologically active domain of LPS is lipid A (27) and the induction of early-phase tolerance in animal models and cellular tolerance to LPS at the level of the macrophage has been shown to be lipid A-dependent (reviewed in Johnston and Greisman [5]). Our data show that R-form LPS induces hyporesponsiveness for S-form LPS and vice versa. This result is expected if adaptation to LPS described here is a lipid A-dependent event.

Studies of Nathan and his colleagues (28, 29) and Pabst et al. (30) have documented changes in macrophage phenotype that occur with LPS concentrations of < 1 ng/ml; the results

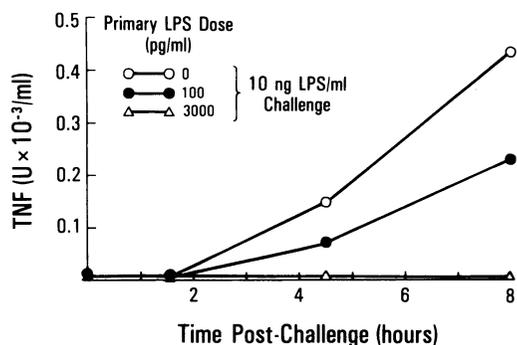


Figure 10. Kinetics of TNF response to 0111:B4 LPS challenge (10 ng/ml) in control PEM or PEM adapted by preexposure for 9 h to primary doses of 100 or 3,000 pg/ml of 0111:B4 LPS. Conditioned medium was harvested 0–8 h after addition of the challenge LPS dose for assay of TNF activity.

reported here display a sensitivity to LPS significantly below these levels. Despite different experimental systems all of these recently reported effects of trace amounts of LPS on macrophage phenotype may reflect programmed responses that serve to limit host cell injury while maintaining bacterial killing mechanisms during gram-negative septicemia. The potential importance of these phenomena is highlighted by the recent report of Rothstein and Schreiber (31) showing the devastating effects of simultaneous injection of LPS and TNF.

The present studies have been limited to induction of TNF by LPS. Whether adaptation to LPS effects the expression of other LPS-induced monokines is not established at this time. Ultimately it will be important to understanding how monokines such as interleukin-1 (IL-1) or interleukin-6 (IL-6) are affected by adaptation. Recent studies have shown that IL-1 and TNF production may be differentially regulated in cells of the monocyte/macrophage lineage (32). Moreover, the recent report of Loppnow et al. (33) studying LPS structure/function relationship for IL-1 expression in human mononuclear cells demonstrated the predominance of lipid A-dependent induction although weak IL-1 inducing activity was reported for core oligosaccharides of LPS. Finally, several cytokines including TNF have been shown to induce or amplify expression of other monokines including IL-1 and IL-6 (34).

Mechanism of LPS-induced adaptation its own effects. Several different mechanisms need to be considered to explain LPS-induced adaptation to its own action including inhibition of TNF gene transcription or translation independent of the stimulus, an increase the rate of degradation of TNF mRNA, or a change in the recognition of LPS by the PEM resulting in diminished initiation of signal transduction pathways for TNF expression.

The result of treating PEM with the primary LPS dose is to decrease sensitivity to LPS. In contrast, when heat-killed *S. aureus* is used to stimulate LPS-adapted cells the amounts of TNF produced are either greater than or equal to TNF production by control (no primary LPS dose) cells. These findings support the contention that adaptation does not result from production of inhibitors of TNF gene transcription or translation that are stimulus-independent.

Here we also show that the marked change in sensitivity of LPS induced by the primary LPS dosage does not appear to depend upon TNF synthesis during the 9-h period when the primary LPS dose is present. Thus TNF or products induced

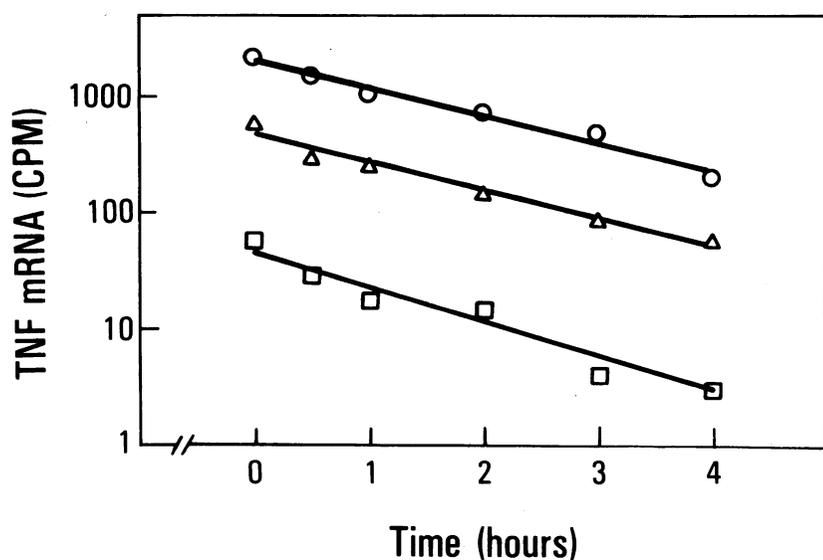
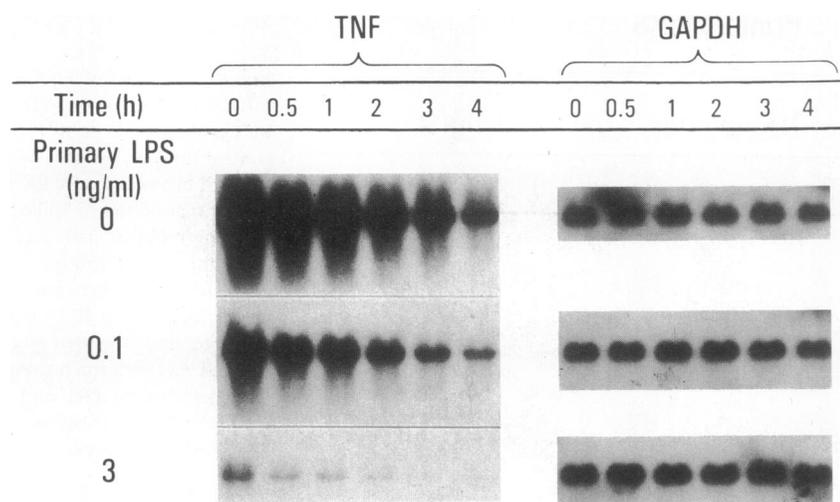


Figure 11. Determination of TNF mRNA half-life in PEM that were adapted to Re595 LPS. PEM were exposed to 0 (○), 0.1 (△), or 3 (□) ng of Re595 LPS/ml for 9 h, washed, and challenged with 100 ng of Re595 LPS/ml. Actinomycin D (5 μg/ml) was added 2 h after challenge, and at various times (0–4 h) after addition of actinomycin D, the conditioned medium was harvested, total cellular RNA was prepared, and Northern blot analysis was performed using ³²P-labeled rabbit TNF and GAPDH cDNA probes as described in Methods. The amounts of mRNA present on the blots were quantitated using a radioactivity scanner, and plotted as shown to determine the TNF mRNA half-life.

by TNF are not likely to be acting as feedback inhibitors of cell function.

Eicosanoids, most notably compounds of the PGE₂ series, have been shown to both inhibit (18, 19) and enhance (24, 35) LPS-induced macrophage responses. Experiments with the cyclooxygenase inhibitor indomethacin used at concentrations

that inhibited PGE₂ production > 95% showed no effect on the induction of adaptation to LPS. This result rules out a significant contribution by prostanoids in adaptation.

Table V. TNF mRNA Half-life in LPS-adapted PEM

Primary LPS dose (ng/ml)	mRNA <i>t</i> _{1/2}		
	0	0.1	3
	<i>h</i>		
Experiment 1	1.3	1.3	1.0
Experiment 2	1.4	1.3	1.8

PEM were incubated 9 h (37°C, 5% CO₂) in the presence or absence of Re595 LPS (primary dose) followed by washing and challenge with 100 ng of LPS/ml. Actinomycin D was added to the cultures 2 h after challenge, and macrophages were harvested at various times 2–6 h after challenge for quantitation of TNF mRNA.

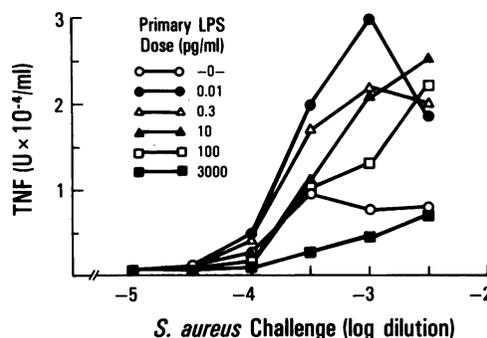


Figure 12. Induction of TNF by *S. aureus* in Re595 LPS-adapted PEM. PEM were exposed to various doses of Re595 LPS (0–3,000 pg/ml) for 9 h, washed, and challenged with various dilutions of heat-killed *S. aureus*. Maximal induction of TNF in control macrophages (○) was observed with 1,000-fold diluted *S. aureus*, corresponding to ~ 50 bacterial cells per macrophage.

The presence of the AUUUA sequence motif in the 3'-untranslated region of the TNF gene provides sequences that may control mRNA stability (36); for example, a ribonuclease recognizing this motif has been suggested to be of importance in regulating TNF mRNA stability (22). The primary LPS dose could either induce or increase the activity of such nucleases or other intracellular proteins that recognize the AUUUA motif and shorten the half-life of TNF mRNA. The role that increased degradation of TNF mRNA plays in adaptation can be tested directly using the inhibitor of RNA synthesis, actinomycin D, comparing TNF mRNA half-life in LPS adapted and control PEM. The data that we show here obtained from actinomycin D experiments argues against a shortening of TNF mRNA half-life being an important feature of LPS-adapted cells.

While several previous reports (6, 37–40) have described the phenomenon of LPS-induced hyporesponsiveness or tolerance to its own effects in macrophages, limited information is available about the mechanisms. An exception to this is the work of Larsen and Sullivan (41) where measurement of colony stimulating factor production by human monocytes was used to better characterize LPS-induced hyporesponsiveness to its own effects. These investigators suggested that the hyporesponsiveness observed in their system resulted from reduced binding and/or impaired processing of LPS. Recognition of LPS implies that macrophages have a specific receptor for LPS that is involved in signal transduction. Although little is known about the identity of a plasma membrane receptor for LPS, several candidates have been identified using cross-linking and ligand-blotting techniques with radiolabeled LPS (42, 43). Current studies are being directed toward more fully defining relationships between LPS-induced adaptation and binding of LPS to macrophages.

Acknowledgments

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