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Endotoxin-Induced Tumor Necrosis Factor Alpha Synthesis in Murine Embryo Fibroblasts

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Murine embryo fibroblasts (MEF) were found to secrete tumor necrosis factor (TNF) in response to stimulation with endotoxin. Endotoxin-induced TNF production by MEF was inhibited by cycloheximide. However, reversal of the effect of this inhibitor on protein synthesis results in TNF being secreted in amounts equivalent to those produced by endotoxin-induced MEF not treated with cycloheximide. Actinomycin D treatment of MEF blocked the production of endotoxin-induced TNF. Maximal production of TNF required MEF gene transcription during the first 6 h of incubation with endotoxin. To determine whether endotoxin-induced TNF alpha (TNF-α) and/or TNF beta were produced by MEF, cDNA was synthesized from the total RNA isolated from endotoxin-induced MEF and amplified by the polymerase chain reaction in the presence of oligonucleotide primers specific for each cytokine. On the basis of the polymerase chain reaction analysis, it was determined that TNF-α mRNA levels were increased in endotoxin-induced MEF. Thus, production of TNF-α by fibroblasts in response to the endotoxin component of bacterial cell walls is likely to contribute to the expression of TNF-mediated effects occurring in fibroblast-rich tissues infected with gram-negative bacteria.

Tumor necrosis factor (TNF) is produced by the host in response to gram-positive or gram-negative bacterial infections (3, 12, 13, 19, 22, 34). Several lines of evidence have established that TNF mediates antibacterial activity. For example, the administration of exogenous preparations of pure TNF protects mice against challenge with any one of a number of different bacteria, including *Listeria monocytogenes* (13), *Escherichia coli* (6), and *Salmonella typhimurium* (25). Again, the use of anti-TNF immunoglobulin G (IgG) to inhibit the action of endogenously produced TNF has established the importance of this cytokine in host antibacterial resistance by showing that it causes exacerbation of murine listeriosis (12, 13), mycobacteriosis (19), and salmonellosis (23).

Macrophages produce TNF in response to stimulation with either gram-positive or gram-negative bacteria (2, 9, 10, 15). Moreover, macrophages produce TNF after exposure to bacterial cell wall components such as endotoxin (1) or lipoarabinomannan (4). Macrophages are not the only cells that produce TNF. On the contrary, there is mounting evidence that other cells of nonlymphoid and nonmyeloid origin are also capable of producing TNF in response to bacteria or to bacterial endotoxin (15, 20, 32). For example, murine embryo fibroblasts (MEF) produce TNF when incubated with the gram-positive bacterium *L. monocytogenes* (15). In this article, we report that MEF can also be induced to produce TNF alpha (TNF-α) in response to stimulation with endotoxin.

MATERIALS AND METHODS

MEF cultures. Primary MEF cultures were established from AB6F1 (A/Tru × C57BL/6Tru) embryos (11). Six to nine embryos of ~16 days of gestation, were aseptically removed from the uterus and placed in a 100-mm-diameter petri dish containing 10 ml of phosphate-buffered saline (pH 7.4) (PBS) and 0.25% trypsin (GIBCO Laboratories, Grand Island, N.Y.) and finely minced with scissors. The tissue fragments were collected by filtration or sterile gauze, washed with PBS, and placed in a trypsinization flask with 40 ml of trypsin solution and agitated for 1 h at room temperature. The trypsin digest was passed through sterile gauze to remove tissue pieces. Cells in the filtrate were pelleted by low-speed centrifugation, the trypsin was decanted, and the cell pellet was suspended in 100 ml of complete RPMI 1640 medium (GIBCO) containing 5% (vol/vol) fetal bovine serum (GIBCO) and 0.25% gentamicin sulfate (GIBCO). Twenty-five milliliters of the cell suspension was placed in 75-cm^2 plastic tissue culture flasks, which were then incubated at 37°C in a humidified 5% CO_2 environment. The next day, the tissue culture medium was aspirated and fresh medium was added to the flasks, which were then incubated until the cells reached confluency. Confluent cells were trypsinized and split 1:3 at each passage level. At the second passage level, some of the cells were frozen for future use. In the experiments reported in this article, cells were used at the sixth to ninth passage levels. At these passage levels, the cells were uniformly fibroblasts on the basis of morphological characteristics (11, 33). Moreover, at these passage levels, the cells neither phagocytized colloidal carbon nor internalized sheep erythrocytes that were opsonized with anti-sheep erythrocyte IgG through Fc receptor-mediated phagocytosis, suggesting the absence of macrophages from the MEF cultures. MEF were seeded at 10^5 cells in 2 ml of complete RPMI 1640 medium into 35-mm-diameter plastic tissue culture dishes and routinely used 2 to 3 days later.

Reagents. Unless specified otherwise, *Salmonella enteritidis* endotoxin extracted by the Boivin trichloroacetic acid procedure and purchased from Difco Laboratories (Detroit, Mich.) was used in the experiments reported. The phenol-extracted *S. enteritidis* endotoxin preparations were also purchased from Difco. Stocks (1 mg/ml) of these bacterial cell wall preparations were made in PBS, sterilized by filtration through a 0.45-μm-pore-size filter, and stored at −20°C. One hour before use, the endotoxin preparations were suspended in complete RPMI 1640 medium at the concentrations stated in the text and placed in a 37°C water

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bath. Lyophilized preparations of sterile actinomycin D were purchased from Calbiochem (La Jolla, Calif.). Immediately prior to use, the actinomycin D was reconstituted in complete RPMI 1640 medium. Stock preparations (100 μg/ml) of cycloheximide (Sigma Chemical Co., St. Louis, Mo.) were prepared in complete RPMI 1640 medium, sterilized by filtration through a 0.45-μm-pore-size filter, and stored at −20°C.

TNF assay. The assay used for the quantitation of TNF cytotoxic activity on actinomycin D-treated murine L929B cells has been described previously in detail (14). The TNF cytotoxicity titer (measured in units per milliliter) is defined as the reciprocal value of the highest serial twofold dilution of sample that causes 50% or more lysis of actinomycin D-treated L929B cells. Included in each assay was an internal laboratory recombinant murine TNF (rMuTNF) standard which was calibrated against the World Health Organization human rTNF standard (86/659) prepared by the National Institute for Biological Standards and Control (Hertfordshire, England).

An anti-TNF neutralization assay (14) using different anti-TNF IgG preparations to characterize the antigenic properties of the TNF produced by MEF was performed. In addition to a polyclonal anti-MuTNF IgG which was raised in rabbits by immunization with pure rMuTNF-α (30), two rat anti-MuTNF monoclonal antibodies (produced by hybridomas MP6-XT3.11 and MP6-XT22 which were the kind gift of John Abrams [DNAX, Palo Alto, Calif.]) were employed in the TNF neutralization assays. The anti-TNF antibody neutralization titer (measured in units per milliliter) is defined as the reciprocal of the highest twofold serial dilution of anti-TNF antibody that, when mixed with an equal volume of test sample having 20 U of TNF cytotoxicity, neutralizes 50% or more of the cytotoxic activity of the sample on actinomycin D-treated L929B cells (14).

RNA preparation. Adherent MEF monolayers (5×10^6 cells) in plastic tissue culture flasks (75 cm^2) were washed once with Mg^{2+}- and Ca^{2+}-free PBS and then treated with 2 ml of denaturing solution (4 M guanidinium isothiocyanate, 25 mM sodium citrate [pH 7.0], 0.5% sarcosyl, 0.1 M 2-mercaptoethanol). Cells were lysed, and cellular material was denatured by gently rocking the flask for 1 min. Aliquots (500 μl) were transferred to microcentrifuge tubes (VWR, Philadelphia, Pa.) for RNA extraction (5) by the procedure described in the RNA isolation kit from Stratagene (catalog no. 200345). RNA was stored in sterile diethylpyrocarbonate-treated glass-distilled water at −70°C.

Oligonucleotide primers. Sequence-specific primers for TNF-α, TNF-β, and β-actin DNA amplification were obtained from Clontech (Palo Alto, Calif.). The internal TNF-α oligonucleotide probe used in Southern blot analysis was an antisense oligomer (5′-CAG CCA CTC CAG CTG CTC 3′) synthesized by Operon (Alameda, Calif.).

Amplification method. Cytokine gene expression was detected by using the procedure in the GeneAmp RNA PCR kit from Perkin-Elmer Cetus (Norwalk, Conn.) with modifications. Briefly, cDNA synthesis was carried out in a total volume of 20 μl in the presence of random hexamers (2.5 μM), deoxynucleoside triphosphates (dNTPs; 1 mM each), magnesium chloride (5 mM), 1× polymerase chain reaction (PCR) buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3]), RNase inhibitor (1 U/μl), Moloney murine leukemia virus reverse transcriptase (2.5 U/μl), and 1 μg of total cellular RNA. All samples were incubated in a GeneAmp 9600 thermal cycler (Perkin-Elmer Cetus) at 42°C for 15 min, 99°C for 5 min, and 5°C for 5 min. For subsequent DNA amplification, the volume of each sample was brought up to 100 μl. The concentration of PCR buffer was kept at 1×, while the concentration of magnesium chloride was changed to 2 mM, and those of dNTPs were changed to 0.2 μM each. Finally, sequence-specific 5′ and 3′ PCR primers (0.1 μM each) and *Taq* polymerase (2.5 U) were added. Each PCR cycle consisted of a denaturation step of 15 s at 95°C and a single annealing and extension step of 1 min at 60°C. The PCR cycling was interrupted after 20, 25, 30, and 35 cycles to determine the extent of amplification for each sample. Aliquots (5 μl) of the PCR were analyzed by electrophoresis on 1.4% agarose gels, and DNA was visualized by ethidium bromide staining.

TABLE 1. Endotoxin-induced MEF production of a cytotoxic factor

S. enteriditis cell wall prepn[a]	Concn of prepn (μg/ml)	MEF present or absent	Cytotoxic activity[b] of 8-h culture medium
TCA-extracted endotoxin[c]	0.5	+	3.0 ± 0.0
	5.0	+	4.0 ± 0.0
	50.0	+	4.0 ± 0.0
	50.0	−	<2.0 ± 0.0
Phenol-extracted endotoxin	0.5	+	4.7 ± 0.6
	5.0	+	4.0 ± 0.0
	50.0	+	4.0 ± 0.0
	50.0	−	<2.0 ± 0.0
Phenol-extracted and column-purified endotoxin	0.5	+	4.0 ± 0.0
	5.0	+	4.0 ± 0.0
	50.0	+	4.0 ± 0.0
	50.0	−	<2.0 ± 0.0

[a] MEF cultures (35 mm) were incubated with 1 ml of medium containing the stated concentrations of the *S. enteriditis* endotoxin preparations for 8 h.
[b] Mean ± standard deviation (log_2 units per milliliter), $n = 3$.
[c] TCA, trichloroacetic acid.

Southern transfer analysis. DNA was transferred to GeneScreen membranes (Dupont, Boston, Mass.) by using standard procedures (28). To determine whether the PCR product obtained with the TNF-α-specific primers was indeed the result of amplifying genuine TNF-α sequence, filters were hybridized to an end-labeled ^{32}P-oligonucleotide probe corresponding to a region of the TNF-α sequence which is internal to the location of the PCR primers (26). Filters were also hybridized to a 540-bp PCR β-actin probe labeled with [α-^{32}P]dCTP by using a random-primed DNA labeling kit purchased from Boehringer Mannheim (Indianapolis, Ind.).

RESULTS

Endotoxin-induced MEF TNF production. MEF were incubated with preparations of *S. enteriditis* endotoxin for 8 h, after which the culture media were assayed for cytotoxicity on actinomycin D-treated L929B cells. Table 1 shows that for the range of concentrations tested (0.5 to 50 μg/ml), the different endotoxin preparations induced similar amounts of cytotoxic activity. Therefore, it seems reasonable to conclude that the lipopolysaccharide moiety of endotoxin was responsible for inducing MEF to secrete the factor that is cytotoxic for actinomycin D-treated L929B cells. This possibility is supported by the observation that the inhibitor of lipopolysaccharide activity, polymyxin B sulfate, diminished the capacity of the phenol-extracted and purified endotoxin

TABLE 2. Serological characterization of cytotoxic activity produced by MEF

Cytotoxic prepn	Neutralizing titer[a] (U/ml) of anti-TNF IgGs for cytotoxic activity		
	Rabbit polyclonal anti-TNF IgG	Rat monoclonal anti-TNF IgG1	
		XT 22	XT 3.11
Endotoxin-MEF medium[b]	4,915	13,107	614
L. monocytogenes-MEF medium[c]	3,277	6,554	410
rMuTNF-α[d]	6,554	6,554	205

[a] The anti-TNF antibody neutralization titer is defined as the reciprocal of the highest twofold serial dilution of antibody that, when mixed with an equal volume of test sample having 20 U of cytotoxic activity, neutralizes 50% or more of the cytotoxic activity on actinomycin D-treated L929B cells.

[b] Endotoxin (5 μg/ml)-containing medium collected after 12 h of incubation with MEF.

[c] MEF cultures (35 mm) were incubated with *L. monocytogenes* (10 CFU per MEF) for 2 h in 0.5 ml of antibiotic-free medium, after which the cultures were washed three times and reincubated with fresh medium (1 ml) containing gentamicin sulfate (0.5 μg/ml). After 24 h of incubation, the culture medium was collected, rendered sterile by filtration, and assayed for cytotoxic activity on actinomycin D-treated L929B cells.

[d] The rMuTNF-α (30) was used as the immunogen to raise the rabbit anti-TNF IgG referred to in this table.

preparation to induce MEF production of TNF by 80% (results not shown).

Studies from this laboratory established that TNF is produced by MEF during incubation with *L. monocytogenes*, a gram-positive bacterium (15). Therefore, to determine whether TNF is also responsible for the cytotoxic activity of the culture medium from endotoxin-treated MEF, anti-TNF IgG neutralization assays were performed. In Table 2 it can be seen that the titers of a polyclonal anti-TNF IgG and two monoclonal anti-TNF IgG1 preparations which neutralize the cytotoxic activity present in the media of MEF incubated with endotoxin or *L. monocytogenes* did not differ more than threefold from the neutralizing titer of the respective anti-TNF IgGs when they were tested against the cytotoxic activity of pure rMuTNF-α. Therefore, on the basis of the results of the anti-TNF IgG neutralization assays, it is concluded that MEF produce TNF in response to stimulation with endotoxin. It must be noted that while these anti-TNF IgG preparations were raised against murine TNF-α, they also cross-react and neutralize the cytotoxic activity of murine TNF-β (27a). However, evidence showing that TNF-α is responsible for the cytotoxic activity will be presented later (see below).

Kinetics of TNF release from endotoxin-induced MEF. Cultures of MEF were incubated with medium containing 5 μg of endotoxin per ml. At the end of the indicated time intervals (Fig. 1), the culture medium was collected and the MEF were replenished with fresh medium containing endotoxin and incubated until the end of the next designated interval, when the procedure was repeated. The amounts of endotoxin-induced TNF secreted by MEF during successive time intervals over a 24-h period are shown in Fig. 1. Endotoxin-induced TNF production by MEF began after 2 h of incubation. The greatest rate of endotoxin-induced TNF secretion by MEF occurred during the first 4 to 6 h of incubation. The data presented in Fig. 1 also show that the continuous exposure of MEF to endotoxin results in TNF secretion for at least 18 h.

Temporal relationship between endotoxin-induced MEF mRNA synthesis and TNF production. Cultures of MEF were

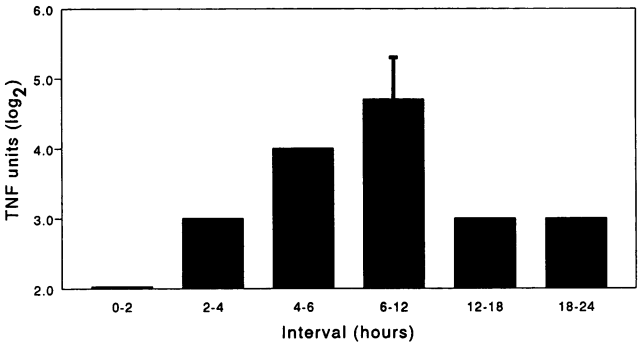

FIG. 1. Kinetics of endotoxin-induced TNF release from MEF. Triplicate MEF cultures (35 mm) were incubated with 1 ml of fresh medium at the start of each designated interval. At the end of each interval, the medium was collected and assayed for TNF (mean $\log_2$ units ± standard deviation).

treated with actinomycin D at progressive times after the onset of incubation with endotoxin to determine the effect of this inhibitor of DNA-dependent RNA synthesis on TNF production. The results of this experiment are presented in Table 3, where it can be seen that the addition of actinomycin D to the MEF culture medium during the first 2 h of incubation with endotoxin totally suppressed TNF production. A partial inhibition of TNF production occurred when actinomycin D was added to MEF cultures at 4 h. The addition of actinomycin D to MEF cultures at either 6 or 8 h after the beginning of incubation with endotoxin results in almost no reduction in TNF production. Therefore, it can be concluded that mRNA synthesis required for TNF production by MEF is largely completed by 6 h after the onset of incubation with endotoxin.

Effect of protein synthesis inhibition on MEF TNF production. The effect of cycloheximide on endotoxin-induced TNF production by MEF was examined by incubating MEF cultures with cycloheximide (5 μg/ml) and endotoxin for 4 h. The culture medium was then removed, and the monolayers were washed three times and reincubated with medium alone for an additional 8 h. The amounts of TNF produced by MEF in the presence and after the removal of cycloheximide and endotoxin are given in Table 4, which shows that in the presence of cycloheximide, endotoxin-induced TNF production by MEF was completely inhibited. However, after the

TABLE 3. Temporal relationship between DNA-dependent RNA transcription and endotoxin-induced MEF TNF production

MEF treatment[a]		TNF activity[b] in 12-h culture medium
Inducing agent	Time actinomycin D added (h)	
None		<2.0 ± 0.0
Endotoxin, 5 μg/ml		5.0 ± 0.0
	0	<2.0 ± 0.0
	2	<2.0 ± 0.0
	4	2.3 ± 0.0
	6	4.3 ± 0.6
	8	4.7 ± 0.6

[a] MEF cultures (35 mm) were incubated at 0 h with 1 ml of medium containing the designated concentration of endotoxin. Actinomycin D was added to sets of MEF cultures, at the times indicated, to a final concentration of 1 μg/ml. Culture media were collected 12 h after the onset of incubation with endotoxin (0 h).

[b] Mean ± standard deviation ($\log_2$ units per milliliter), $n = 3$.

TABLE 4. Effect of cycloheximide treatment on endotoxin-induced MEF production of TNF

MEF treatment[a]		TNF activity[b] produced	
0 to 4 h	4 to 12 h	0 to 4 h	4 to 12 h
Medium	Medium	<2.0 ± 0.0	<2.0 ± 0.0
Endotoxin	Medium	5.0 ± 0.0	4.3 ± 0.6
Endotoxin + cycloheximide	Medium	<2.0 ± 0.0	5.0 ± 0.0
Cycloheximide	Medium	<2.0 ± 0.0	<2.0 ± 0.0

[a] At 0 h, MEF cultures were incubated with 1 ml of medium or medium containing cycloheximide (5 μg/ml). Endotoxin was added to the indicated groups of cultures to a final concentration of 5 μg/ml. Four hours later, the culture media were collected, the MEF cultures were washed three times, and then all cultures were incubated for an additional 8 h with medium alone. Data presented are representative of four independent experiments.

[b] Mean ± standard deviation (log_2 units per milliliter), $n = 3$.

removal of the cycloheximide, the MEF synthesized an amount of TNF equivalent to that produced by MEF not treated with cycloheximide during the 4-h incubation period with endotoxin.

Analysis of TNF gene expression in endotoxin-induced MEF. PCR analysis was employed to determine whether TNF-α and/or TNF-β (lymphotoxin) mRNA levels were increased in MEF after exposure to endotoxin. cDNA was synthesized from total RNA isolated from untreated MEF and MEF that were incubated with endotoxin for 2 or 4 h. The cDNA preparations were then amplified by PCR for 20, 25, 30, and 35 cycles by using primers that were specific for TNF-α, TNF-β, and β-actin. β-Actin was amplified to verify that initial RNA concentrations were equivalent among the various samples.

PCR products were not discernible after 20 PCR cycles (1.3×10^5-fold amplification). After an additional five cycles (another 20-fold amplification), β-actin DNA became evident in all MEF preparations (Fig. 2); however, TNF-α DNA was only evident in the MEF samples incubated with endotoxin for 2 and 4 h. After an additional five cycles, TNF-α DNA became detectable in the sample prepared from the control MEF that were not incubated with endotoxin. After 35 cycles, TNF-α was clearly evident in the unstimulated control, and we also began to detect PCR products of differing sizes, which probably represent nonspecific products arising from primer annealing to mismatched sequences.

These data indicate that incubation of MEF with endotoxin for 2 h resulted in increased TNF-α mRNA levels (compare lanes 1 and 4). To verify that the sequence amplified between the two PCR primers is indeed the TNF-α target sequence, we hybridized a Southern transfer blot of the PCR gels to an oligonucleotide probe specific for TNF-α sequences internal to the primers originally used for PCR. It is clear from Fig. 2D that the PCR product corresponding to TNF-α hybridizes to the internal oligomer. This confirms that the 692-bp PCR product observed was generated from TNF-α mRNA. It is also clear from Fig. 2 that the 278-bp TNF-β-specific PCR product remains undetectable within the time period examined. However, to ensure that failure to detect TNF-β mRNA in MEF cells was not due to an inability of the primers to yield a PCR product, a preparation of RNA isolated from the pancreas of transgenic mice expressing the TNF-β gene under the control of insulin gene transcriptional regulatory elements (27) was amplified in tandem with RNA isolated from MEF incubated for 4 h with endotoxin. It can be seen in Fig. 3 that a TNF-β-specific

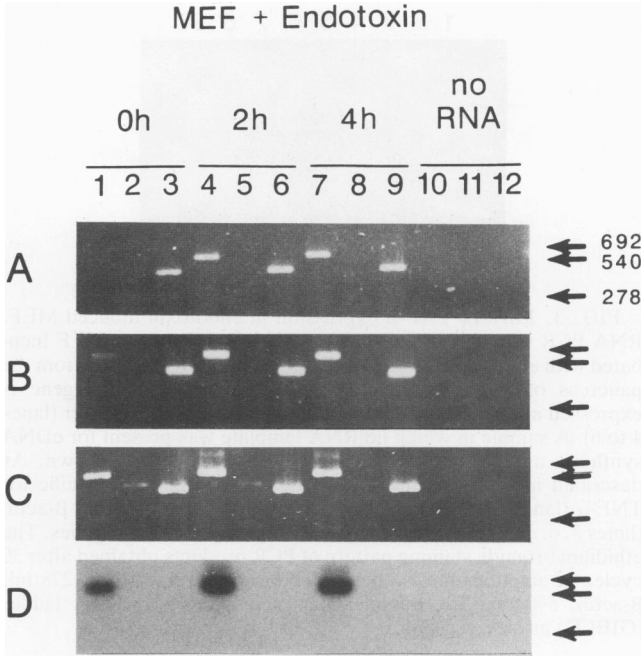

FIG. 2. Analysis of TNF mRNA levels in endotoxin-stimulated MEF. RNA PCR was performed on total RNA isolated from untreated MEF (lanes 1 to 3) and MEF incubated with endotoxin for 2 h (lanes 4 to 6) and 4 h (lanes 7 to 9). By using oligonucleotides (Clontech amplimers) specific for TNF-α (lanes 1, 4, 7, and 10), a 692-bp PCR product is obtained, while for TNF-β (lanes 2, 5, 8, and 11), a PCR product of 278 bp is predicted. Finally, β-actin (lanes 3, 6, 9, and 12) amplification results in a 540-bp PCR product. Panels A through C show ethidium bromide staining of PCR products isolated on a 1.4% agarose gel after amplifications of 25, 30, and 35 cycles, respectively. Panel D shows the Southern blot analysis of the PCR products obtained after 35 cycles of amplification that are shown in panel C by using an end-labeled oligonucleotide primer specific for TNF-α which is internal to the PCR primers. A sample in which no RNA template was present for cDNA synthesis and subsequent PCR is shown as a control.

PCR product was not present in the MEF RNA but was clearly detectable in the RNA preparation isolated from the pancreas of the transgenic mice. These results establish that TNF-β is not produced by MEF in response to endotoxin stimulation during the time period examined. It should be noted that on the basis of scanning densitometry of Southern blots, β-actin levels for all MEF samples are roughly equivalent (data not shown).

DISCUSSION

TNF produced during bacterial infections can be either detrimental or beneficial to the host. In the first instance, the presence of very large quantities of TNF in the systemic circulation, as occurs during overwhelming bacterial infections, is associated with systemic toxicity and the pathological state of cachexia (31). In the second instance, the production of physiological amounts of TNF in infected tissues has been shown to be essential in host defense against a variety of bacterial infections (12, 13, 19, 22, 23, 34).

Macrophages are probably an important source of TNF during bacterial infections (2, 9, 10, 15). However, there is a growing body of evidence that many different cell types can

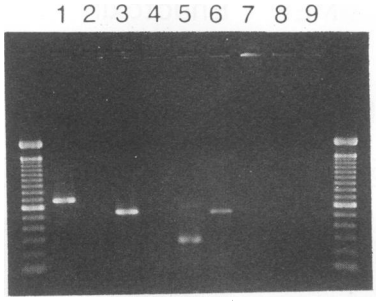

FIG. 3. Lack of TNF-β expression in endotoxin-induced MEF. RNA PCR was performed on total RNA isolated from MEF incubated with endotoxin for 4 h (lanes 1 to 3) and RNA isolated from the pancreas of transgenic mice (27) in which the TNF-β gene is expressed and under the control of the rat insulin II promoter (lanes 4 to 6). A sample in which no RNA template was present for cDNA synthesis and subsequent PCR (lanes 7 to 9) is also shown. As described in Materials and Methods, oligonucleotides specific for TNF-α (lanes 1, 4, and 7), TNF-β (lanes 2, 5, and 8), and β-actin (lanes 3, 6, and 9) were used to detect expression of these genes. The ethidium bromide staining pattern of PCR products obtained after 30 cycles of amplification is shown. TNF-α, 692 bp; TNF-β, 278 bp; β-actin, 540 bp. The outside lanes show a 100-bp DNA ladder (GIBCO) as size markers.

also produce TNF in response to bacteria or bacterial components. For example, it was reported that human smooth muscle cells (SMC) (32) and keratinocytes (20) can secrete TNF after exposure to endotoxin. Consistent with these reports, experiments presented herein show that TNF-α appears in the culture medium of mouse embryonal fibroblasts during incubation with endotoxin. This is in keeping with the ability of these fibroblasts to produce a variety of cytokines, including TNF, when infected with *L. monocytogenes* (11, 15). In view of the ubiquity of fibroblasts in all tissues, the localized production of TNF by these cells in response to invading gram-positive or gram-negative bacteria may prove important in TNF-dependent host antibacterial defense mechanisms.

PCR analysis showed that although TNF was not detected in the culture medium of MEF in the absence of endotoxin, TNF-α mRNA was present at very low levels in these cells. It is not known whether this TNF-α mRNA is due to low-level constitutive transcription of the TNF-α gene, an existing pool of stable TNF-α mRNA, or the result of induction of the TNF-α gene due to low levels of TNF-inducing agents (e.g., endotoxin) present in the culture medium. However, TNF-α mRNA has also been reported to exist in unstimulated macrophages and certain tumor cell lines (1, 17, 29). PCR analysis also revealed that 2 h after exposure to endotoxin, the amount of TNF-α mRNA present in MEF was greatly enhanced over that found in untreated MEF. However, TNF cytotoxic activity was not detected in the culture medium until 2 to 4 h after exposure of MEF to endotoxin. It is not known whether this increase in TNF mRNA levels is due to the induction of gene transcription and/or the increase in TNF mRNA stability. The finding that the addition of actinomycin D to MEF cultures at the end of the first 2 h of incubation with endotoxin completely prevented the release of TNF into the culture medium suggests that endotoxin may also induce the transcription of other mRNAs which encode for factors that are required in the translation of the TNF-α mRNA and/or the secretion of TNF. Thus, endotoxin-induced TNF production by MEF, like endotoxin-induced TNF production by macrophages, may be regulated at both transcriptional and posttranscriptional levels (1, 35).

Endotoxin-induced TNF production was inhibited by cycloheximide. However, removal of both cycloheximide and endotoxin from MEF cultures after a 4-h incubation period resulted in the secretion of TNF, thereby suggesting that the endotoxin-induced increase of TNF-α mRNA occurs in spite of the inhibition of protein synthesis. Indeed, this was expected, given that endotoxin-induced TNF gene transcription occurs in SMC when protein synthesis is blocked by cycloheximide (32). In fact, the amounts of endotoxin-induced TNF-α mRNA in SMC at the end of a 4-h cycloheximide-mediated blockade of protein synthesis were greatly elevated (superinduced) over the amounts of TNF-mRNA that were present in SMC treated with only endotoxin. In contrast, the amount of endotoxin-induced TNF released from MEF after the reversal of protein synthesis inhibition approximated that produced by endotoxin-induced MEF not treated with cycloheximide. Thus, cycloheximide treatment of MEF did not result in a superinduction of endotoxin-induced TNF production. In view of this finding and the observation that TNF was not secreted by MEF after a 4-h treatment with only cycloheximide (Table 4), it would appear that the posttranscriptional regulation of TNF synthesis and secretion is not under the control of a labile repressor molecule.

It is becoming apparent that cell types of nonmyeloid or nonlymphoid origin are capable of producing cytokines that have roles in inflammation and/or the generation of immunity. For example, human fibroblasts derived from neonate foreskins are reported to produce interleukin-6 (IL-6) when incubated with endotoxin (16). Moreover, TNF induces MEF to secrete IL-6 (15). Again, endotoxin and TNF induce the production of IL-1 in cultures of SMC (32). Therefore, it is possible that a variety of different cells are potential in vivo sources of TNF, which is capable of inducing in an autocrine or paracrine manner the localized production of IL-1 and a cascade of other cytokines, including IL-6 (8, 18, 21). In view of the demonstrated importance of TNF and IL-1 in inflammation (7, 24), the early production of these cytokines by various nonprofessional phagocytic cell types such as fibroblasts may serve to provide, or amplify, the initial signal that results in the recruitment and focusing of host cells having antibacterial function (e.g., polymorphonuclear leukocytes) to infectious foci in tissues and organs.

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