

# Cachectin/Tumor Necrosis Factor- $\alpha$ Formation in Human Decidua

## Potential Role of Cytokines in Infection-induced Preterm Labor

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### Abstract

This study was conducted as part of an investigation to evaluate the hypothesis that bacterial toxins (LPS or lipoteichoic acid), acting on macrophage-like uterine decidua to cause increased formation of cytokines, may be involved in the pathogenesis of infection-associated preterm labor. We found that cachectin/tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) was synthesized and secreted into the culture medium by human decidual cells and explants in response to treatment with LPS. LPS treatment also caused an increase in PGF<sub>2 $\alpha$</sub>  production by decidual cells and explants. In amnion cells in monolayer culture, TNF- $\alpha$  stimulated PGE<sub>2</sub> formation, and TNF- $\alpha$  was cytostatic (inhibited [<sup>3</sup>H]thymidine incorporation into DNA) but not cytolytic in amnion cells. TNF- $\alpha$  was not detectable (< 0.34 ng/ml) in the amniotic fluid of normal pregnancies at midtrimester or at term before or after the onset of labor ( $n = 44$ ); but TNF- $\alpha$  was present at concentrations between 2.8 and 22.3 ng/ml in amniotic fluids of 4 of 20 pregnancies with intact membranes complicated by preterm labor (< 34 wk gestational age). LPS was present in 10 of the 20 amniotic fluids of preterm labor pregnancies, including all four in which TNF- $\alpha$  was present. Bacteria were identified in only one of the four LPS-positive, TNF- $\alpha$ -positive fluids. Cytokine formation in macrophage-like decidua may serve a fundamental role in the pathogenesis of preterm labor, including increased prostaglandin formation and premature rupture of the membranes.

### Introduction

The greatest single cause of newborn morbidity and mortality is preterm birth (1, 2); but regrettably, the sequence of biomolecular events that leads to the initiation of human parturition, whether at term or preterm, is not known. Appreciable numbers of pregnancies that culminate in preterm labor are complicated by infectious processes that involve intrauterine tissues (namely, extraembryonic fetal membranes or decidua or both [3–10]) or else extrauterine maternal tissues (e.g., as with pyelonephritis, pneumonia, or peritonitis [10–16]). This unfortunate association of infection and preterm labor may

prove to be fortuitous, however, because it may constitute a model system to evaluate key biomolecular processes involved in spontaneous parturition at term. Therefore, an understanding of the processes involved in infection-associated preterm labor could lead to the development of a means to prevent premature birth and also to understand selected biomolecular processes of spontaneous parturition at term.

There is considerable evidence in support of the likelihood that prostaglandins (principally, PGF<sub>2 $\alpha$</sub> ) serve as the mediators (uterotonins) of the myometrial contractions that are characteristic of labor in women (see also 17, 18). This evidence can be summarized as follows: (a) the administration of prostaglandins (intraamniotically, vaginally, or intravenously) in human pregnancy leads to the onset of myometrial contractions and delivery at any stage of gestation; (b) the administration of inhibitors of prostaglandin synthesis to pregnant women is associated with prolonged gestation and an increase in the time interval for intraamniotic hypertonic saline induction of abortion; and (c) the concentrations of prostaglandins in amniotic fluid and in maternal plasma and urine are increased during parturition. The levels of PGE<sub>2</sub> and PGF<sub>2 $\alpha$</sub>  in amniotic fluid increase strikingly during labor, as does the level of 13,14-dihydro-15-keto-PGF<sub>2 $\alpha$</sub>  ([PGFM],<sup>1</sup> the major metabolite of PGF<sub>2 $\alpha$</sub> ) (19, 20). The concentration of PGFM in maternal plasma also increases (21, 22); on the other hand, there is no demonstrable increase in the levels of metabolites of PGE<sub>2</sub> in maternal plasma during labor (23, 24). Thus, PGF<sub>2 $\alpha$</sub>  is believed to be the principal uterotonic agent, whereas increased fetal membrane production of PGE<sub>2</sub> also is coincident with labor. The fetal membranes (amnion and chorion laeve) produce PGE<sub>2</sub> almost exclusively; but the decidua is a tissue site of PGF<sub>2 $\alpha$</sub>  formation (25, 26). It is likely, therefore, that the PGF<sub>2 $\alpha$</sub>  in amniotic fluid and the PGFM present in maternal plasma in increased amounts during labor arise in decidua.

We hypothesized that LPS, originating in microorganisms in uterine or extrauterine tissues, may act on macrophage-like decidua to initiate a series of events that culminate in preterm labor, possibly together with premature rupture of the fetal membranes. Evidence in support of this hypothesis is as follows: (a) more than 40 years ago, it was shown that the administration of LPS to pregnant animals causes abortion or premature parturition (27, 28); (b) in LPS-treated animals, abortion or preterm delivery is associated with decidual hemorrhage and necrosis (27); (c) LPS acts on monocytes-macrophages to cause the production of prostaglandins (29), cachectin/tumor necrosis factor (TNF- $\alpha$ ) (30), and IL-1 (31); and (d) the decidua is enriched in monocytes-macrophages

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1. *Abbreviations used in this paper:* LAL, *Limulus* ameobocyte lysate; mEGF, murine epidermal growth factor; PGFM, 13,14-dihydro-15-keto-prostaglandin F<sub>2 $\alpha$</sub> ; TNF- $\alpha$ , cachectin/tumor necrosis factor- $\alpha$ .

(32); in fact, the decidual cell per se is macrophage-like in several functional characteristics (33).

This investigation was conducted to evaluate selected tenets of this proposition. Specifically, we sought (a) to ascertain whether LPS acts on decidua to cause TNF- $\alpha$  production; (b) to evaluate LPS or TNF- $\alpha$  action in amnion and decidua to stimulate prostaglandin formation; (c) to evaluate the cytostatic or cytotoxic effects of TNF- $\alpha$  on amnion (the fetal membrane bathed by the amniotic fluid in vivo); and (d) to determine whether TNF- $\alpha$  was present in amniotic fluid of pregnancies complicated by preterm labor.

## Methods

**Tissues and cell and explant cultures.** Human placentae were obtained aseptically at the time of elective cesarean section at term and were used as a source of decidual tissue, amnion tissue, and villous trophoblast tissue. The decidual tissue was removed from the chorion laeve by sharp dissection. Villous trophoblast tissue was removed from the placenta with care to avoid areas of fibrosis or calcification. The tissues were rinsed in culture medium to remove blood. Decidual tissue or pieces of placental villous trophoblast tissue ( $\sim 1 \text{ mm}^3$ ) were placed in explant culture (approximately five tissue pieces were placed in 35-mm-diam plastic culture dishes with 3 ml culture medium; or alternatively, three tissue pieces were placed in 16-mm-diam culture wells with 1.5 ml culture medium). The explants were maintained in Waymouth enriched culture medium that contained fetal bovine serum (10%, by volume) for 12–16 h before the commencement of experiments. The decidual and villous placental tissue explants are heterogeneous in cellular composition.

Decidual cells were isolated from decidua vera tissue according to the method of Gurpide and colleagues (34) by limited enzymatic digestion and separation on Percoll gradients. The cells were plated in plastic 24-well plates and allowed to replicate to confluence in primary monolayer culture.

Amnion tissue was separated from chorion laeve by blunt dissection. The amnion tissue was minced and cells were dispersed by enzymatic digestion as described previously (35). The isolated amnion cells were suspended in Ham F12:Dulbecco MEM (1:1, by vol) that contained fetal bovine serum (10%, by vol) and antibiotic-antimycotic solution (1%, by vol). The amnion cells were plated in plastic culture dishes (24-well plates), maintained at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air, and allowed to replicate in monolayer to confluence.

Cytotrophoblasts were isolated from term villous trophoblast tissue by limited enzymatic digestion and separation on Percoll gradients according to the method of Strauss and colleagues (36). The cytotrophoblasts were placed in culture in plastic 24-well plates for 16 h before commencement of the experiment.

Endometrial tissue was collected from the uteri of premenopausal women after hysterectomy that was conducted for reasons other than endometrial disease. Informed consent for the use of tissues was obtained from each woman before the surgical procedure. The consent form and protocol used were approved by the Institutional Review Board of this university. Endometrial stromal cells were prepared as described (37) and as modified in our laboratory (38). Briefly, endometrial tissue was minced into small pieces ( $\sim 1 \text{ mm}^3$ ), and the minced tissue was incubated at 37°C for 20–40 min in HBSS that contained 25 mM Hepes, 200 U/ml penicillin, 200  $\mu\text{g}/\text{ml}$  streptomycin, 1 mg/ml collagenase (134 U/mg), and 0.08 mg/ml DNase (1,950 Kunitz units/ml). The dispersed endometrial stromal cells were separated from endometrial glands by filtration through a 75- $\mu\text{m}$  wire sieve. The endometrial stromal cells (in the filtrate) were pelleted and washed twice by centrifugation (400 g, 10 min) and suspension in culture medium that did not contain collagenase or DNase. The cells

were suspended finally in Waymouth enriched culture medium (Waymouth MB752/1 medium, MEM vitamins, MEM amino acids, non-essential amino acids, antibiotics-antimycotics [38]) that contained fetal bovine serum (10%, by volume). The cells were plated in plastic culture dishes (24-well plates, 16-mm-diam wells) at a density of  $\sim 200,000$  cells/well per ml of culture medium. The endometrial stromal cells were maintained in a tissue culture incubator at 37°C in a humidified atmosphere of CO<sub>2</sub> (5%) in air. Experiments were conducted with endometrial stromal cells in confluent primary monolayer culture or after first or second passage.

Explants or cells in monolayer culture were treated with (or without) LPS (3, 30, or 100 ng/ml) for various times. LPS was dissolved in the appropriate culture medium for the cells or explants to be tested. In other experiments, cells or explants were treated with TNF- $\alpha$  ( $10^{-9}$ – $10^{-11}$  M) or murine epidermal growth factor (mEGF) (15 ng/ml) that was solubilized in the appropriate culture medium.

**Northern analysis of TNF- $\alpha$  mRNA.** Explants of human uterine decidua or placental villous trophoblast tissues were maintained in culture for 16 h in Waymouth enriched medium that contained fetal bovine serum (10%, by volume). Thereafter, the culture medium was removed and replaced with Waymouth enriched medium that contained fetal bovine serum (10%, by volume) with or without LPS (*Escherichia coli* 055:B5; 100 ng/ml); the explants were incubated for 2 h. At the end of the incubation period, the culture media were collected for quantification of TNF- $\alpha$  and prostaglandins and the tissue explants were collected to determine the specific content of mRNA for TNF- $\alpha$ . Total RNA was extracted and mRNA for TNF- $\alpha$  was evaluated by Northern analysis as described (39).

**Quantification of prostaglandins and evaluation of cell replication.** Amnion cells in primary monolayer culture, or decidual tissues in explant culture were treated with recombinant human TNF- $\alpha$  ( $10^{-9}$  M), mEGF (15 ng/ml), or both for various times. In experiments with amnion cells, cell replication was evaluated as follows: 2 h before the end of the 24-h incubation period, an 0.5-ml aliquot of culture medium was removed (for quantification of prostaglandins) and [<sup>3</sup>H]-thymidine (0.5  $\mu\text{Ci}/\text{well}$ ) was added. At the end of the incubation period, the medium was removed and discarded. The cells were scraped from the dishes and aliquots were applied to 3-mm (Whatman Inc., Clifton, NJ) filters. DNA was precipitated onto the filter with TCA. The incorporation of [<sup>3</sup>H]thymidine into TCA-precipitable material (DNA) was quantified by liquid scintillation spectrometry. Prostaglandins in culture media were quantified directly by use of specific radioimmunoassays for PGE<sub>2</sub> and PGF<sub>2 $\alpha$</sub> . Antisera were purchased from Advanced Magnetics (Boston, MA). The intra- and interassay coefficients of variation for radioimmunoassay of PGE<sub>2</sub> are 8.7 and 8.7%, respectively, and those for radioimmunoassay of PGF<sub>2 $\alpha$</sub>  are 12.6 and 13.2%, respectively. Cell protein content was quantified by the method of Lowry et al. (40).

**Amniotic fluids.** Amniotic fluids were collected (for diagnostic reasons unrelated to the conduct of this study) from singleton pregnancies before rupture of the fetal membranes by transabdominal amniocentesis at midtrimester, by transuterine amniocentesis at the time of cesarean section conducted at term before or after the onset of labor, or by needle aspiration of the intact fetal membranes per vagina during labor. Amniotic fluids also were collected (in the same manner) from 20 singleton pregnancies complicated by preterm (< 34 wk) labor. In all cases, the amniotic fluids were collected directly into nontoxic, nonpyrogenic, sterile polypropylene syringes (Monoject, Brunswick Co., St. Louis, MO). Specially prepared, endotoxin-free glassware was used for processing and storage of the amniotic fluids; the glassware was made endotoxin-free by autoclaving and heat treatment at 180°C for 3 h. An aliquot of the amniotic fluid was transferred to a port-a-cul tube for evaluation of anaerobic and aerobic bacteria. The remainder of the amniotic fluid sample was centrifuged at 300 g; the supernate was sterilized by filtration (pore diameter 0.45  $\mu\text{m}$ , then 0.22  $\mu\text{m}$ ) and stored at 4°C. Previously, we found that inadvertent contamination of amniotic fluid by bacterial endotoxin was avoided by careful use of this method of collection and processing (41).

The absence or presence of LPS in amniotic fluids was assessed by use of the *Limulus* amoebocyte lysate (LAL) gelation method as described previously (41). The sensitivity of the LAL gelation assay for identification of LPS in amniotic fluid is  $\sim 0.016$  endotoxin units (relative to *E. coli* 055:B5 LPS standardized to the U. S. Pharmacopeia Reference Standard Endotoxin) (41). (This assay, like other LAL-based assays for LPS, is useful specifically for the detection of the endotoxin. LPS cannot be quantified with respect to biopotency by use of LAL-based assays because there is wide variation in the effectiveness of various endotoxins to activate LAL.)

**Quantification of TNF- $\alpha$ .** TNF- $\alpha$  in amniotic fluids and conditioned culture medium (from decidual or placental explants, decidual cells, endometrial stromal cells, cytotrophoblasts, or amnion cells) was assayed by use of WEHI 164.1 cells or L929 cells and the method of Ortaldo et al. (42). WEHI 164.1 cells were maintained in monolayer culture in RPMI 1640 culture medium that contained 10% fetal bovine serum (vol/vol), 0.1 mM glutamine, and 30  $\mu$ g/ml gentamicin (referred to as RPMI culture medium). L929 cells were maintained in Eagle's MEM that contained 10% horse serum (vol/vol),  $1\times$  nonessential amino acids (Gibco Laboratories, Grand Island, NY), 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin (referred to as EMEM culture medium). For use in the assay, the cells (of either cell line) were removed from the plate by brief trypsinization. The cells were labeled by incubation at 37°C for 1 h with  $^{51}\text{Cr}$  (100  $\mu\text{Ci}$  per  $10^7$  cells, 250 mCi/mg Cr) in RPMI or EMEM culture medium. The cells were washed by centrifugation and suspension; and, finally, the  $^{51}\text{Cr}$ -labeled WEHI 164.1 cells or L929 cells were suspended in RPMI or EMEM culture medium ( $10^6$  cells/ml) that contained actinomycin D (2 or 10  $\mu$ g/ml, respectively). 0.1-ml aliquots of the cell suspension were transferred to round-bottomed wells of 96-well microtiter plates and incubated at 37°C for 16 h together with 0.1-ml aliquots of test solutions (i.e., amniotic fluid or conditioned culture medium). At the end of the incubation period,  $^{51}\text{Cr}$  released into the culture medium was quantified (in 0.1-ml aliquots) in a gamma spectrometer (Packard Instruments, Downers Grove, IL). The total releasable  $^{51}\text{Cr}$  was quantified by treatment of the cells with Zapoglobin (Coulter Diagnostics, Hialeah, FL). Spontaneous release of chromium-51 was determined for cells treated with culture medium that contained no test agents. The percent cell lysis after treatment with test agents was computed as follows: % cell lysis = (cpm released by test agent) - (cpm released spontaneously)/(cpm released by Zapoglobin) - (cpm released spontaneously)  $\times 100$ . The specificity of the chromium-51 release assay for TNF- $\alpha$  was assessed by neutralizing the activity with a specific MAb to TNF- $\alpha$ . 2  $\mu$ l anti-TNF- $\alpha$  (9 ng/ml) was mixed with an 0.5-ml aliquot of the test solution or standard solution before adding 0.1 ml of the solution to the microtiter plate. All assays were conducted in replicates of four. The least amount of TNF- $\alpha$  detectable was  $4 \times 10^{-4}$  pmol. To ensure the validity of the assay for TNF- $\alpha$  in amniotic fluid and culture media, experiments were conducted in which recombinant human TNF- $\alpha$  ( $10^{-11}$ – $10^{-9}$  M) was added to the fluids or media before conducting the assay; in all cases, the TNF- $\alpha$  measured was within 10% of the expected value, and in all cases, the cytolytic activity attributable to the TNF- $\alpha$  was neutralized by use of the MAb. Results obtained in the  $^{51}\text{Cr}$  release assay for TNF- $\alpha$  were corroborated by use of ME180 cells and the crystal violet assay (43).

To evaluate the cytostatic or cytolytic effect of TNF- $\alpha$  on human amnion, a similar procedure for trypsinization,  $^{51}\text{Cr}$  labeling, treatment, and quantification were used with confluent amnion cells in primary monolayer culture.

**Materials.** Culture media and fetal bovine serum were purchased from Irvine Scientific (Santa Ana, CA). [ $^3\text{H}$ ]Thymidine (20 Ci/mmol) was purchased from ICN Biochemicals (Irvine, CA).  $^{51}\text{Cr}$  was purchased from New England Nuclear (Boston, MA). Human recombinant TNF- $\alpha$  and mouse MAb to human recombinant TNF- $\alpha$  was a gift from Chiron Corp. (Emeryville, CA). mEGF (culture grade) was purchased from Collaborative Research Corp. (Bedford, MA). LPS (*E. coli* 055:B5) was purchased from Sigma Chemical Co. Percoll was purchased from Pharmacia Fine Chemicals, Piscataway, NJ.

## Results

**Effect of LPS-treatment on TNF- $\alpha$  production by human uterine decidua.** The effect of LPS treatment on TNF- $\alpha$  production by decidual tissue explants was evaluated in two ways: (a) TNF- $\alpha$  in the culture media of decidual explants was quantified by use of assays of cytolytic activity; and (b) the content of mRNA specific for TNF- $\alpha$  in tissue explants after 2 h of LPS-treatment was quantified by Northern analysis. The amount of TNF- $\alpha$  that accumulated in the culture medium of decidual explants during 20 h of treatment with 1  $\mu$ g/ml LPS was  $0.27 \pm 0.01$  pmol/mg tissue protein compared with  $0.05 \pm 0.01$  pmol/mg tissue protein in medium of nontreated (control) decidual explants (Table I). Maximal stimulation of TNF- $\alpha$  production by decidual explants was observed with LPS at a concentration of 1  $\mu$ g/ml (data not shown). In addition, we evaluated the production of TNF- $\alpha$  by decidual cells and endometrial stromal cells (the progenitor cell of the decidual cell) in monolayer culture. Decidual cells produced TNF- $\alpha$  in response to LPS treatment; the amount of TNF- $\alpha$  in the culture media of untreated decidual cells was  $< 0.15$  pmol/mg protein compared with  $1.35 \pm 0.06$  pmol/mg protein in the medium of decidual cells treated with 1  $\mu$ g/ml LPS for 20 h. In contrast, endometrial stromal cells did not produce TNF- $\alpha$  in detectable amounts, even after treatment with LPS (Table I). The increase in production of TNF- $\alpha$  by decidual tissues was accompanied by a striking increase in mRNA for TNF- $\alpha$ . By Northern analysis (Fig. 1), mRNA for TNF- $\alpha$  was apparent in untreated (control) decidual tissue; but, in response to treatment with 100 ng/ml LPS, there was a marked increase in mRNA for TNF- $\alpha$  in this tissue.

In nontreated explants of placental villous trophoblast tissue, mRNA for TNF- $\alpha$  also was detectable, but in an amount appreciably less than that present in untreated decidual tissue; the level of mRNA for TNF- $\alpha$  in placental tissue explants was increased by treatment with LPS but remained significantly less than that in LPS-treated decidual tissue (Fig. 1). The amount of TNF- $\alpha$  in culture media of villous trophoblast explants increased with 1  $\mu$ g/ml LPS (for 20 h) treatment from  $0.03 \pm 0.01$  to  $0.96 \pm 0.05$  pmol/mg protein. LPS treatment (1  $\mu$ g/ml, for 20 h) stimulated TNF- $\alpha$  production by cytotrophoblasts in monolayer culture; the amount of TNF- $\alpha$  in the media of untreated cells was  $0.02 \pm 0.01$  pmol/mg protein and

Table I. Production of TNF- $\alpha$  by Decidual Tissue Explants, Decidual Cells, and Endometrial Stromal Cells Treated with LPS

Experimental model	TNF- $\alpha$ production	
	Control	LPS
	pmol TNF- $\alpha$ /mg protein per 20 h	
Decidual tissue explants	$0.05 \pm 0.01$	$0.27 \pm 0.01$
Decidual cells in monolayer	$< 0.15$	$1.35 \pm 0.06$
Endometrial stromal cells in monolayer culture	$< 0.004$	$< 0.004$

Decidual explants, isolated decidual cells, or endometrial stromal cells were maintained in culture in the presence of 1  $\mu$ g/ml LPS for 20 h. Thereafter, the concentration of TNF- $\alpha$  in the culture media and the amount of cell or tissue explant protein were quantified as described in Methods. Data are expressed as mean  $\pm$  SEM,  $n = 3$ .

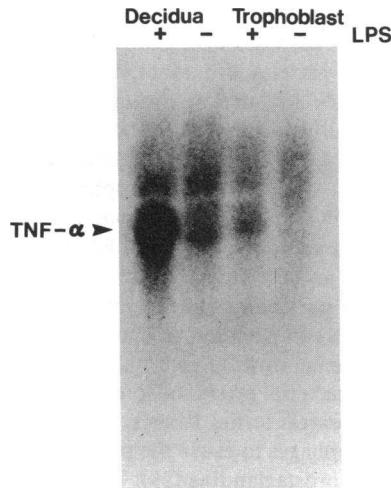


Figure 1. Northern analysis of TNF- $\alpha$  mRNA in decidua (lanes 1 and 2) and villos placental (lanes 3 and 4) tissue explants maintained in organ culture in the absence (-) or presence (+) of 100 ng/ml LPS (*E. coli* 055:B5) for 4 h.

that in media of LPS-treated cells was  $0.07 \pm 0.02$  pmol/mg protein.

**Effect of LPS on PGF<sub>2 $\alpha$</sub>  production by decidua.** PGF<sub>2 $\alpha$</sub>  and TNF- $\alpha$  were quantified in the culture media of decidua explants after treatment with LPS (3 and 30 ng/ml) for 6 h. PGF<sub>2 $\alpha$</sub>  production was increased from  $0.19 \pm 0.11$  ng/mg tissue protein per 6 h in control explants to  $0.55 \pm 0.17$  and  $0.87 \pm 0.25$  ng/mg tissue protein per 6 h in the medium of decidua explants treated with LPS (3 and 30 ng/ml, respectively) (Table II). In this experiment, the amount of TNF- $\alpha$  produced by the untreated explants was below the limit of detection ( $< 0.002$  pmol/mg protein); in response to LPS (3 and 30 ng/ml), TNF- $\alpha$  production increased to  $0.02 \pm 0.002$  and  $0.10 \pm 0.01$  pmol/mg tissue protein.

**Effect of TNF- $\alpha$  on human amnion cell growth and prostaglandin production.** The production of PGE<sub>2</sub> by amnion cells was stimulated by treatment with TNF- $\alpha$  ( $10^{-9}$  M). After treatment for 22 h, the amount of PGE<sub>2</sub> that accumulated in the culture medium of cells treated with TNF- $\alpha$  was increased by three to sevenfold compared with untreated (control) cells (Table III). TNF- $\alpha$  inhibited mitogenesis of human amnion cells maintained in primary monolayer culture as determined by quantification of the incorporation of radiolabeled thymidine into TCA-precipitable material (DNA). In cells treated

Table II. Production of PGF<sub>2 $\alpha$</sub>  and TNF- $\alpha$  by Decidua Explants Treated with LPS

Treatment	PGF <sub>2<math>\alpha</math></sub> production	TNF- $\alpha$ production
	ng/mg tissue protein per 6 h	pmol/mg tissue protein per 6 h
Control	$0.19 \pm 0.11$	ND*
LPS (3 ng/ml)	$0.55 \pm 0.17^{\dagger}$	$0.02 \pm 0.002^{\S}$
LPS (30 ng/ml)	$0.87 \pm 0.25^{\parallel}$	$0.10 \pm 0.01^{\ddagger}$

Decidua explants were maintained in the absence or presence of LPS (3 or 30 ng/ml). After incubation for 16 h, TNF- $\alpha$  and PGF<sub>2 $\alpha$</sub>  in the culture media and cell protein were quantified as described. Data are expressed as mean  $\pm$  SEM,  $n = 3$ .

\* ND, not detectable ( $< 1.8 \times 10^{-15}$  mol/mg protein);  $^{\dagger}$  NS ( $P > 0.05$ ) compared with control;  $^{\S}$   $P < 0.01$ ;  $^{\parallel}$   $P < 0.03$ ;  $^{\ddagger}$   $P < 0.05$ .

Table III. Stimulation of PGE<sub>2</sub> Production by Amnion Cells Treated with TNF- $\alpha$

Experiment no.	PGE <sub>2</sub> production	
	Control	TNF- $\alpha$
	ng PGE <sub>2</sub> /mg protein per 22 h	
1	$3.8 \pm 0.7$	$13.2 \pm 2.7^*$
2	$2.2 \pm 0.2$	$8.4 \pm 0.9^{\dagger}$
3	$7.5 \pm 2.2$	$22.9 \pm 2.2^{\ddagger}$
4	$0.4 \pm 0.05$	$2.7 \pm 0.5^*$

Human amnion cells in primary monolayer culture (confluent) were treated with culture medium that contained TNF- $\alpha$  ( $10^{-9}$  M). After incubation for 22 h, PGE<sub>2</sub> in the culture medium and cell protein were quantified as described in Methods (33). Data are expressed as mean  $\pm$  SEM,  $n = 3$ .

\*  $P < 0.02$ ,  $^{\dagger}$   $P < 0.002$ .

with TNF- $\alpha$  ( $10^{-11}$ ,  $10^{-10}$ , and  $10^{-9}$  M), thymidine incorporation was decreased compared with that in untreated cells (Table IV). The inhibitory effect of TNF- $\alpha$  ( $10^{-11}$ – $10^{-9}$  M) on DNA synthesis in these cells was attenuated, but not abolished, in the presence of EGF (15 ng/ml) (Fig. 2).

To determine whether the effect of TNF- $\alpha$  on amnion cell DNA synthesis was due to cytolysis or cytostasis, a number of studies were conducted. First, the effect of TNF- $\alpha$  in amnion cells was evaluated by use of the  $^{51}$ Cr release assay. At concentrations of  $10^{-11}$ – $10^{-9}$  M, TNF- $\alpha$  did not effect cell lysis (Fig. 3). Moreover, amnion cells (treated with  $10^{-10}$  M TNF- $\alpha$  for 16 h) recovered from the cytostatic effect when the culture medium that contained TNF- $\alpha$  was removed and replaced with medium that contained no additions; the incorporation of [ $^3$ H]thymidine into DNA of cells so treated was identical with that in cells not pretreated with TNF- $\alpha$ . And, as in untreated cells, the TNF- $\alpha$ -pretreated amnion cells responded to mEGF by increased rate of incorporation into DNA (data not shown). Bacterial endotoxin (0.5–100 ng/ml) was not cytostatic in amnion cells as evaluated by [ $^3$ H]thymidine incorporation into DNA after 22 h of treatment; and, amnion cells treated with LPS did not produce TNF- $\alpha$  in detectable amounts.

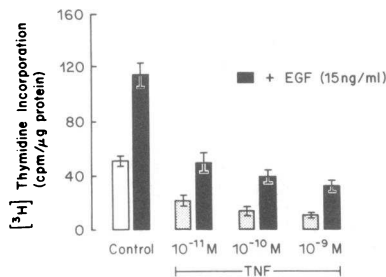
**Identification and quantification of TNF- $\alpha$  in human amniotic fluid.** TNF- $\alpha$  was undetectable ( $< 0.34$  ng/ml) in amniotic fluids obtained at midtrimester of pregnancy ( $n = 10$ ) or at

Table IV. Effect of TNF- $\alpha$  on [ $^3$ H]Thymidine Incorporation into DNA of Amnion Cells

Treatment	[ $^3$ H]Thymidine incorporation
	cpm/ $\mu$ g protein*
Control	$182.6 \pm 18.8$
mEGF (15 ng/ml)	$526.7 \pm 40.0^{\ddagger}$
TNF- $\alpha$ ( $10^{-11}$ M)	$51.8 \pm 1.5^{\ddagger}$
TNF- $\alpha$ ( $10^{-10}$ M)	$19.6 \pm 2.4^{\ddagger}$
TNF- $\alpha$ ( $10^{-9}$ M)	$8.6 \pm 1.2^{\ddagger}$

\* Mean  $\pm$  SEM.

$^{\ddagger}$   $P < 0.001$ , compared with control.



**Figure 2.** Effect of mEGF on inhibition by cachectin/TNF- $\alpha$  of [ $^3$ H]thymidine into DNA of human amnion cells maintained in primary monolayer culture. Confluent amnion cells in monolayer culture were treated with TNF ( $10^{-11}$ – $10^{-9}$  M) in

the absence or presence of mEGF (15 ng/ml) for 24 h. The extent of incorporation of [ $^3$ H]thymidine into TCA-precipitable material was evaluated during the final 2 h of the incubation period. Data are expressed as the mean  $\pm$  SEM ( $n = 6$ ). In the presence of mEGF, [ $^3$ H]thymidine incorporation was increased significantly compared with that in the absence of mEGF ( $P < 0.001$  for control,  $10^{-11}$  M TNF- $\alpha$ ,  $10^{-10}$  M TNF- $\alpha$ ;  $P < 0.002$  compared with  $10^{-9}$  M TNF- $\alpha$ ).

term before ( $n = 20$ ) or after ( $n = 14$ ) the spontaneous onset of labor. The failure of detection of TNF- $\alpha$  in these fluids is believed to be due, in fact, to the absence of TNF- $\alpha$  and not due to the presence of an inhibitor or a substance that interfered with the assay because exogenously added TNF- $\alpha$  was predictably quantifiable. Lysis of the WEHI 164.1 cells to a small extent (equivalent to the lysis effected by 0.4–0.7 ng/ml TNF- $\alpha$ ) was effected by some of these amniotic fluids from normal pregnancies, but the cytolytic activity was not neutralized by anti-TNF- $\alpha$  antibody.

TNF- $\alpha$  was identified in 4 of 20 amniotic fluid samples obtained from pregnancies complicated by preterm ( $< 34$  wk gestation) labor. The levels of TNF- $\alpha$  in these four amniotic fluids were 2.8, 5.4, 21.7, and 22.3 ng/ml; *Fusobacterium sp.* was identified in one of these fluids (that in which TNF- $\alpha$  was 2.8 ng/ml); the other three were sterile. LPS was present in 10 of the 20 amniotic fluids and in each of the four samples in which TNF- $\alpha$  was present in detectable amounts. TNF- $\alpha$  was not detectable in amniotic fluids of two pregnancies in which the women were known to have pyelonephritis.

## Discussion

In pregnancies complicated by infection, whether it be intra-uterine or extrauterine, labor often commences preterm (3–15); moreover, preterm rupture of the fetal membranes is a common accompaniment or antecedent event of preterm labor. And whatever the intermediate processes may entail, prostaglandins, produced in increased amounts by uterine decidua and fetal membranes, are believed to mediate, physiologically, the initiation of labor in women (cf 44). Heretofore,

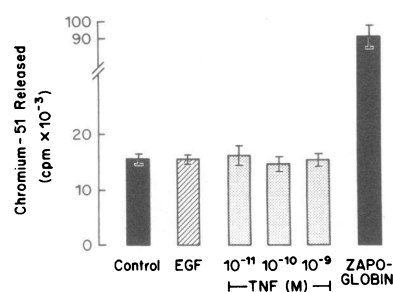
some investigators have suggested that infections, especially those that involve the fetal membranes and contiguous decidua vera, may lead, in some undefined manner, to preterm rupture of the membranes and thence preterm labor. Others have proposed that the liberation of phospholipase A<sub>2</sub> (45) or else phospholipase C (46) by invading bacteria may promote the release of AA from amnion glycerophospholipids and thence the generation of PGE<sub>2</sub>. But these explanations, even if operative, seem inadequate to explain completely the relationships between infection and the preterm onset of labor.

We elected to examine the possibility that infection-associated preterm labor is initiated by activation of macrophage-like decidua in response to the action of a factor(s), namely, bacterial toxins (LPS or other bacterial products), produced by microorganisms in intra- or extrauterine tissues. It is well known that LPS acts on macrophages to cause the production of TNF- $\alpha$  (30), IL-1 (31), and prostaglandins (29). Therefore, we evaluated the hypothesis that LPS acts on decidual tissue to cause the production of TNF- $\alpha$  and that TNF- $\alpha$  acts in amnion or in decidua to alter cell function, namely, cell growth or prostaglandin production or both. To support this hypothesis, there is appreciable evidence that many properties of decidual tissue are macrophage like (32, 33); and decidual activation appears to be synchronous with parturition, whether labor occurs at term or preterm (18).

In this investigation, we found that TNF- $\alpha$  is produced by decidual tissue and by isolated decidual cells in response to treatment with LPS. TNF- $\alpha$  was not produced in detectable amounts by endometrial stromal cells, the progenitors of the decidual cells. Amnion cells did not produce TNF- $\alpha$  in detectable amounts in response to LPS.

We also found that TNF- $\alpha$  is cytostatic, but not cytolytic, in human amnion cells. Amnion cells, therefore, are relatively unique because other normal cells commonly respond to TNF- $\alpha$  as a growth factor or, alternatively, not at all (47–49). Indeed, TNF- $\alpha$ , while cytolytic or cytostatic (48–50) in a number of tumor cells, commonly does not inhibit growth in normal cells. The possibility thus exists that LPS, or some other bacterial factor, acts on macrophage-like decidual cells (or macrophages of decidua or both) to cause the production of TNF- $\alpha$ , which, in turn, may act to inhibit growth of the amnion (a process that may favor rupture of the membranes); TNF- $\alpha$  may also act in vivo, as it does in vitro, to stimulate the production of PGF<sub>2 $\alpha$</sub>  by decidua and PGE<sub>2</sub> by amnion.

A variety of proteins produced in decidua accumulate in the amniotic fluid. For example, decidual prolactin is transferred almost exclusively into amniotic fluid (51). TNF- $\alpha$ , however, was not present in amniotic fluid of normal pregnancies at any stage of gestation or during spontaneous labor at term. But, TNF- $\alpha$  was present in amniotic fluid of 4 of 10 pregnancies complicated by preterm labor in which the membranes were intact and LPS also was present in the amniotic fluid. LPS-stimulated macrophage-like decidual cells, LPS-stimulated macrophages of decidual tissue, or both, thus constitute potential tissue sites of origin of TNF- $\alpha$  in amniotic fluid of pregnancies complicated by infection-associated preterm labor. We presume that LPS entered amniotic fluid in some preterm labor pregnancies by diffusion across the fetal membranes, originating in microorganisms infecting decidua, chorion laeve, or outer amnion. Alternatively, gram-negative microorganisms may have invaded the amniotic fluid but replication of these bacteria may have been inhibited by the anti-



**Figure 3.** Lack of cytolytic action of TNF- $\alpha$  on amnion cells. Cytolysis of amnion cells in primary monolayer culture incubated with mEGF (15 ng/ml), TNF- $\alpha$  ( $10^{-11}$ – $10^{-9}$  M), or Zapoglobin for 16 h was evaluated by use of the  $^{51}$ Cr release assay as described in Methods.

microbial activities of this fluid. In either case, LPS in amniotic fluid is a sensitive index of infection because LPS is not a constituent of normal amniotic fluid at any stage of gestation before or during labor (41).

We cannot be sure that TNF- $\alpha$  was not produced in decidua of some preterm labor pregnancies in which LPS was present in the amniotic fluid but in which TNF- $\alpha$  was not identified. For example, in 6 of 10 amniotic fluids in which LPS was present, TNF- $\alpha$  was not detected. This could mean that TNF- $\alpha$  was not produced or else too little was transferred and accumulated in amniotic fluid to be detected.

In most medical centers, premature rupture of the membranes is found in upwards of two-thirds of pregnancies complicated by preterm labor. Commonly this seems to be the antecedent event in the precipitation of preterm labor. It is possible that TNF- $\alpha$  produced in decidua in these pregnancies acts to promote premature rupture of the fetal membranes. But, this is a difficult proposition to evaluate directly. Studies of residual amniotic fluid after rupture of the membranes is fraught with complexities. Because of the patency of the female reproductive tract, bacterial colonization of the fetal membranes and amniotic fluid after rupture of amnion and chorion could lead to spurious results; namely, infection may have followed rather than preceded membrane rupture. It is for this reason that we chose to confine this initial study to an evaluation of amniotic fluids from pregnancies with intact fetal membranes. Nonetheless, infection leading to TNF- $\alpha$  formation in decidua may be an important intermediate event in pregnancies in which early rupture of the fetal membranes heralds the imminent onset of preterm labor.

In preliminary studies, we have obtained evidence that decidua, as well as endometrial stromal cells, also produce IL-1 (52). We find that the concentration of IL-1 $\beta$  in amniotic fluid increases during spontaneous parturition and increases even more dramatically during preterm labor complicated by infection (53); we also find that IL-1 acts on decidua and endometrial stromal cells to augment strikingly PGF<sub>2 $\alpha$</sub>  formation (52). LPS, acting on decidua thus may cause, as in macrophages, the secretion of both TNF- $\alpha$  and IL-1. Therefore, LPS may act in decidua to provoke those responses known to be associated with preterm birth, namely rupture of membranes, and increased prostaglandin formation.

We suggest that a definition of the biomolecular events involved in infection-associated preterm labor may lead not only to the development of a means for the prevention of some cases of preterm labor but also may provide an opportunity to develop new strategies for the study of the initiation of spontaneous labor in normal pregnancies at term.

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