

Fetal Cytokine Expression *In Utero* Detected by Reverse Transcriptase Polymerase Chain Reaction

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ABSTRACT

Cytokine expression at the maternal-fetal interface is well documented. Some authors have postulated the existence of a bidirectional cytokine signaling mechanism that is critical to the maintenance of normal pregnancy. The role of the fetus (*versus* fetally derived placental tissues) in this process is unknown. Using reverse transcription polymerase chain reaction techniques, we studied paired maternal and fetal samples from 16 pregnancies (including two twin pregnancies) for the presence of tumor necrosis factor- α and IL-1 β mRNA. We demonstrate that mRNA for both of these cytokines can be detected in both

maternal and fetal blood as early as the 21st wk of gestation. These results support a potential role for the fetus in the bidirectional cytokine signaling of pregnancy. (*Pediatr Res* 37: 450-454, 1995)

Abbreviations

PCR, polymerase chain reaction
rtPCR, reverse transcriptase polymerase chain reaction
TNF α , tumor necrosis factor- α

Cytokine expression at the maternal-fetal interface is well documented (1). Developmental changes in levels of TNF- α , IL-1 β , and IL-6 have been described in both placental tissue and amniotic fluid (2), with significant rises in all three occurring at the time of labor. However, the precise role of any of these cytokines in either maintaining normal pregnancy or initiating labor remains unknown. Wegmann and coworkers have suggested a critical role for cytokines in normal pregnancy, both for the maintenance of placental growth (3) and the modulation of maternal immune responses to prevent "rejection" of the semiallogenic conceptus (4). A central feature of Wegmann's hypothesis is that the cytokine interactions that characterize the maternal-fetal relationship are bidirectional and that both maternal and fetal (or fetally derived) tissues and cells participate in mutual regulatory responses (5).

The precise role of the fetus in the putative cytokine regulatory network of pregnancy has not been investigated. Abbas *et al.* (6) have demonstrated the presence of TNF- α in fetuses as early as 12 wk of gestation. TNF- α levels were higher in fetuses of greater gestational age, suggesting that fetal levels of this cytokine parallel those in amniotic fluid. However, al-

though these authors concluded that the TNF- α detected in fetal plasma was of fetal origin and coincided with the development of the fetal monocyte-macrophage system, the precise origin of the fetal plasma TNF- α could not be determined. It is equally possible that the TNF- α detected in fetal plasma was derived from placental or decidual cells, which communicate directly with the fetal circulation.

PCR techniques provide a convenient means of addressing issues related to the fetal cytokine repertoire. Although the presence of a specific cytokine in fetal plasma may not be conclusive evidence that the fetal immune system does secrete or is capable of secreting that cytokine (inasmuch as cytokines may pass from placental tissue to the fetus), the presence of cytokine mRNA in fetal blood cells would support the hypothesis that cytokine expression occurs in the fetus as well as in placental tissue. The small amounts of RNA available in fetal blood specimens obtained by cordocentesis may yield considerable information about fetal cytokine mRNA transcripts and therefore the role of the fetus itself in the bidirectional cytokine signaling proposed by Wegmann *et al.* (5). We used rtPCR to examine maternal and fetal mRNA transcripts in the late second and third trimesters.

METHODS

Patients and patient specimens. Specimens from 16 pregnancies were studied. In two cases, blood from the mother and from each fetus of a twin pregnancy was available for study

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(therefore, 16 mothers and 18 fetuses were studied). Blood was obtained by cordocentesis from fetuses at 21 to 39 wk of gestation after prior written informed consent was obtained from the mothers. The procedure for cordocentesis has previously been described (7). Gestational ages of fetuses and indications for cordocentesis are given in Table 1. Samples were drawn into sterile EDTA tubes and carried to the laboratory. Plasma was separated from cells by centrifugation, and cell pellets were stored at -70°C until used.

Isolation of RNA from whole blood. RNA was isolated from whole blood by a method modified from that described by Chomczynski and Sacchi (8). Two hundred μL of whole blood were placed in 500 μL of denaturing solution consisting of 4.0 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% sodium N-laurosarcosine, and 0.1 M 2-mercaptoethanol, with 20 μg polyinosinic acid used as carrier RNA. Samples were vortexed and 200 μL of 95% ethanol added. Samples were then placed on ice for 5 min and subjected to centrifugation in a microfuge at $10\,000 \times g$ for 15 min at 4°C . Supernates were discarded and pellets dissolved in 150 μL of denaturing solution and 15 μL of 2.0 M sodium acetate, pH 4.2. Solubilized pellets were then incubated on ice for 10 min with an equal volume of phenol-chloroform solution (10:4 vol/vol) and subjected to centrifugation at $10\,000 \times g$ for 15 min at 4°C . The aqueous phase was saved and precipitated with an equal volume of isopropyl alcohol, with samples incubating at -20°C for 30 min. After incubation, samples were centrifuged at $10\,000 \times g$ for 15 min at 4°C and supernatants discarded. Pelleted RNA was washed once with 75% ethanol and dissolved in sterile water with 0.1% diethylprocarbinat. RNA was either frozen at -20°C or used immediately for reverse transcription reactions.

Reverse transcription of RNA to DNA. Reverse transcription of RNA to DNA was accomplished with Moloney murine leukemia virus reverse transcriptase and random hexamer primers using commercially available reagents (GeneAmp, Perkin Elmer Cetus, Norwalk, CT). Conditions for reverse transcription were 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 5.0

mM MgCl_2 , 1.0 mM each deoxynucleotriphosphates, 2.5 μM random hexamers, 2.5 U/ μL reverse transcriptase, and 1.0 U/ μL RNase inhibitor with 2.0 μL of target RNA in a total volume of 20 μL . Reactions were performed in a single three-step cycle: 42°C for 15 min, 99°C for 5 min, and 5°C for 5 min. DNA generated from this process was either stored at -20°C or used immediately for PCR experiments to detect specific cytokine mRNA.

PCR and agarose gel electrophoresis to detect cytokine mRNA. Oligonucleotide primers for TNF- α , IL-1 β , and β -actin were obtained from a commercial source (Clontech, Palo Alto, CA). Sequences of the 5' and 3' primers for each cytokine are given in Table 2. Amplification reactions were accomplished with *Thermus aquaticus* DNA polymerase (2.0 U per reaction) in 50 mM KCl, 10 mM Tris-HCl, 1.5 mM MgCl_2 , 0.2 mM deoxynucleotriphosphates, 0.4 μM 5' and 3' primers, with 5.0 μL of target DNA in a volume of 50 μL . Cycling was performed at 94°C for 45 s for denaturation, 60°C for 45 s for annealing, and 72°C for 2 min for elongation for 35 cycles. This program was linked to single 72°C (7 min) and 4°C cycles. Samples were then solubilized in 6 \times buffer consisting of 0.25% bromphenol blue, 0.25% xylene cyanol, and 30% glycerol and subjected to electrophoresis in 1.8% agarose-ethidium bromide gels. Gels were examined for bands of appropriate molecular weight and compared with a positive control generated from a cDNA fragment provided with the primer sets encompassing the sequences amplified in the PCR process. β -actin was also used as a positive control and to determine the efficiency of RNA isolation and cDNA amplification.

Southern blot analysis of amplified PCR products. In selected cases, verification of the identity of the PCR product was performed using Southern blot analysis using commercially available cDNA probes for the sequences amplified in the PCR reactions (Clontech, Palo Alto, CA). Probes were labeled with horseradish peroxidase using commercially available reagents (ECL chemiluminescence detection system, Amersham, Arlington Heights, IL). PCR products were transferred to nylon membranes by capillary action overnight in 1.5 M NaCl and 0.5 M NaOH. Prehybridization and hybridization of the labeled probes were performed exactly according to the manufacturer's specifications. Signal was generated from the labeled probes by the addition of the detection reagent provided with the labeling kit. Membranes were exposed to Kodak XAR film for times varying from 30 s to 5 min, and the resultant luminograms were examined for bands corresponding to the positive control generated as described above.

RESULTS

Results from these experiments are summarized in Table 3. The agarose gel from a typical PCR analysis of maternal and fetal blood samples is shown in Figure 1. TNF- α mRNA was assayed in 16 maternal-fetal pairs. TNF- α mRNA was detected in all 18 fetal blood samples and in 15 of the 16 maternal samples. Although ethidium bromide-stained bands for fetal TNF- α were generally more prominent than bands from maternal samples, this was probably due to the more efficient

Table 1. Patients, gestational age, and indications for cordocentesis

Patient	Gestational age (wk)	Indications
1	23	Polyhydramnios
2	24	Renal dysplasia
3	29	Polyhydramnios
4	29	Primary CMV infection
5	31	Rh incompatibility
6	39	Primary CMV infection
7	33	Premature rupture of membranes
8	36	Intrauterine growth retardation
9	26	Intrauterine growth retardation
10	36	Discordant twins
11	21	Discordant twins
12	22	Primary varicella infection
13	23	Intrauterine growth retardation
14	32	Preterm labor
15	23	Polyhydramnios
16	34	Preterm labor

CMV, cytomegalovirus.

Table 2. Sequences of primers used for reverse transcriptase PCR

β -Actin	
Upstream (5')	5' ATC TGG CAC CAC ACC TTC TAC AAT GAG CTG CG 3'
Downstream (3')	5' CGT CAT ACT CCT GCT TGC TGA TCC ACA TCT GC 3'
IL-1 β	
Upstream (5')	5' ATG GCA GAA GTA CCT AAG CTC GC 3'
Downstream (3')	5' A CAC AAA TTG CAT GGT GAA GTC AGT T 3'
TNF α	
Upstream (5')	GAG TGA CAA GCC TGT AGC CCA TGT TGT AGC A 3'
Downstream (3')	GCA ATG ATC CCA AAG TAG ACC TGC CCA GAC T 3'

Table 3. Results of cytokine rtPCR assays*

Patient	TNF α		IL-1 β	
	Mother	Fetus	Mother	Fetus
1	+	+	ND	ND
2	+	+	ND	ND
3	+	+	ND	ND
4	+	+	Neg	+
5	+	+	+	+
6	+	+	+	+
7	+	+	+	+
8	Neg	+	+	+
9	+	+	+	+
10†	+	+/+	+	+/+
11	+	+	+	+
12	+	+	+	+
13	+	+	+	+
14†	+	+/+	Neg	Neg/Neg
15	+	+	Neg	Neg
16	+	+	Neg	Neg

ND, not done; Neg, negative.

* All samples were positive for β -actin.

† Twin pregnancy.

RNA isolation from fetal blood (which contains more nucleated cells in a given volume than maternal blood), inasmuch as β -actin bands tended to be brighter in these same samples (Fig. 1A). The β -actin band in the mother with undetectable TNF- α mRNA was strongly positive, confirming that the RNA isolation, reverse transcription, and PCR were as efficient as in the other maternal samples.

PCR analysis for IL-1 β , which was examined in 13 pregnancies, showed a different pattern (Fig. 2). In three pregnancies (including one of the twin pregnancies), IL-1 β was clearly negative. One pregnancy showed discordance in IL-1 β expression between mother and fetus, with the fetus showing a faint IL-1 β band and the mother negative. In two other maternal-fetal pairs, faint bands for IL-1 β were produced by PCR. In seven pregnancies, IL-1 β was clearly positive.

Expression of IL-1 β may have been related to fetal exposure to viral infection, inasmuch as mRNA for this cytokine was clearly positive in the 22-wk fetus (and mother) with maternal varicella infection (patient 12), whereas weak bands were seen in both of the infants exposed to primary maternal cytomegalovirus infection. Because no attempt was made to make the PCR assays quantitative or even semiquantitative, no conclusions can be drawn regarding the level of TNF- α or IL-1 β mRNA expression and specific clinical situations (e.g. intrauterine growth retardation).

DISCUSSION

The discovery that TNF- α , IL-1 β , and IL-6 are expressed at the maternal-fetal interface has generated considerable interest in their role in pregnancy and the initiation of labor. Wegmann *et al.* (5) have suggested that normal pregnancy requires a bidirectional, intercommunicating cytokine network that includes the maternal immune system and fetally derived placental tissues, especially placental trophoblasts (4). Whether the fetus plays any role in this network is unknown. The finding of TNF- α in fetal plasma as described by Abbas *et al.* (6) was supportive of a role for the fetus in this process. However, monocytes obtained from both term and preterm infants postnatally have been shown to have little constitutive expression of either IL-1 β or TNF- α , although such cells will respond to bacterial lipopolysaccharide to produce IL-1 β and, to a lesser extent, TNF- α (9).

We have presented strong evidence for fetal cytokine mRNA expression *in utero* as early as 21 wk of gestation. The specific cytokines detected, TNF- α and IL-1 β , are most commonly expressed in cells of the monocyte/macrophage lineage and suggest that this portion of the immune system is already well developed by the second trimester. Why these cells transcribe low levels of TNF- α and IL-1 β mRNA during fetal life is unclear. Although it is possible that low levels of mRNA for these cytokines may be detected in resting monocytes in normal infants or children (a question that we are currently addressing), it is also possible that expression of these cytokines reflects fetal participation in the so-called cytokine network of pregnancy. Alternatively, it could reflect the fact that the monocyte-macrophage system in the fetus is in an activated state due to rapid growth and differentiation. A similar hypothesis has been suggested for fetal T cells, a high percentage of which express the activation marker CD38 (7).

We have considered the possibility that the TNF- α and IL-1 β mRNA amplified from cordocentesis specimens may have come from a few contaminating amniotic fluid cells. However, levels of fetal mRNA were, if anything, higher than the amount of mRNA from maternal blood. It therefore seems very unlikely that the cytokine mRNA amplified from the fetal samples came from anywhere but fetal blood.

We believe that PCR technology will be an important tool in the understanding of the immunobiology of the fetus and pregnancy, particularly in investigating the role of cytokines. Because most cytokines are secreted and act locally, and because expression of many cytokines is accompanied by expression of soluble receptors and inhibitors, there are serious

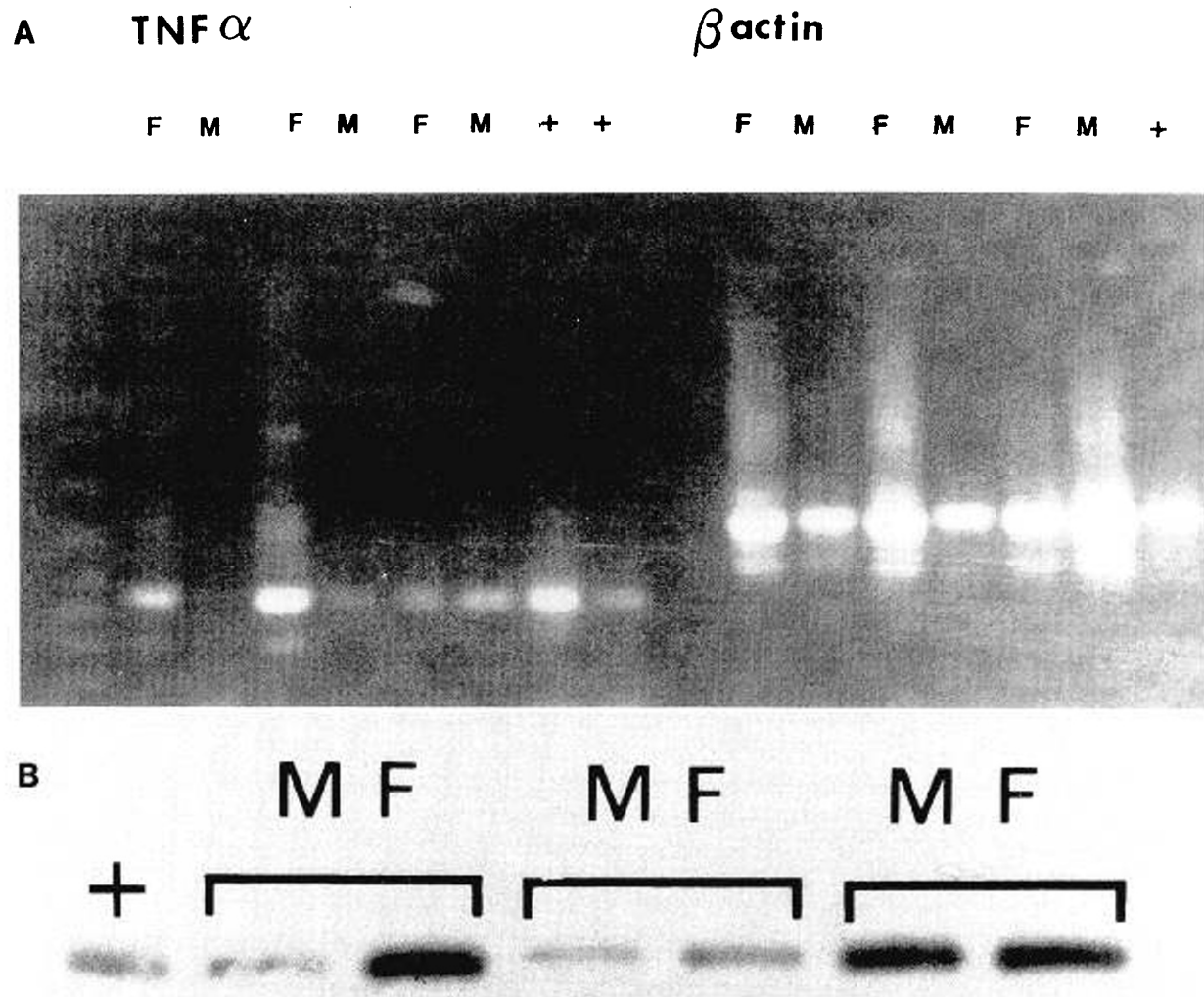


Figure 1. A, Ethidium bromide–stained agarose gel showing amplified products from three maternal fetal-pairs (patients 1, 2, and 3). TNF- α is shown on the left and β -actin on the right. Fetal (F) and maternal (M) samples are designated. Positive control lanes are designated “+”. Although signals from the fetal TNF- α samples seem to be stronger than those from maternal samples, this may have been caused by the larger amount of RNA isolated from fetal whole blood: β -actin bands were also slightly brighter in fetal samples. B, Southern blot analysis of DNA products generated from rtPCR analysis for TNF- α in three maternal-fetal pairs (Patients 14, 15, and 16).

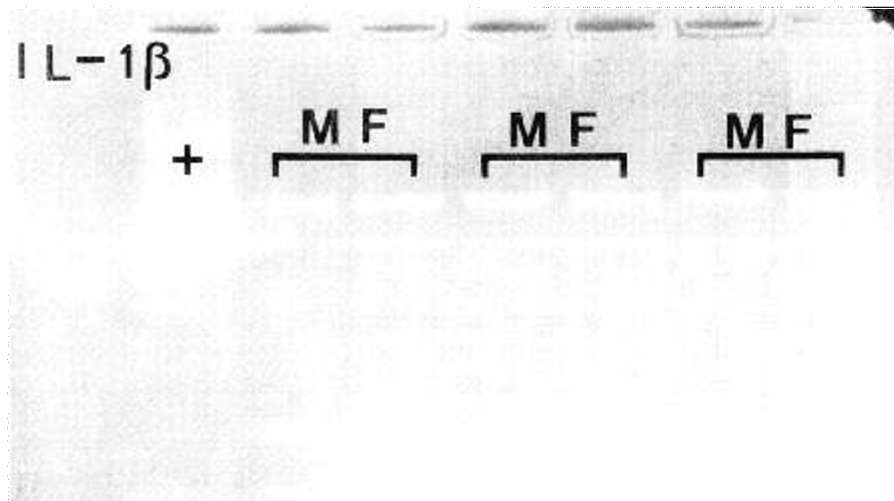


Figure 2. Representative ethidium bromide/agarose gel showing rtPCR analysis for IL-1 β in blood samples from three maternal-fetal pairs. Shown here are the three maternal-fetal pairs that were most strikingly positive for IL-1 β (Patients 11, 12, 13). Maternal (M) and fetal (F) samples as well as positive controls (+) are designated.

pitfalls in the measurement of these proteins in plasma by either bioassay or immunologic methods. The use of rTPCR, however, provides a sensitive, specific, and even quantitative method (10) for examining cytokine expression *in vivo* or *in vitro*. These techniques, coupled with our increasing ability to sample fetal tissue *in vivo*, provide the opportunity for significant advances in our understanding of fetal immunobiology in the near future.

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REFERENCES

1. Kauma S, Matt D, Strom S, Eierman D, Turner T 1990 Interleukin 1 β , human leukocyte antigen HLA-DR α , and transforming growth factor β expression in endometrium, placenta, and placental membranes. *Am J Obstet Gynecol* 163:1430–1437
2. Opsjøn S-L, Wathen NC, Tingulstad S, Wiedswang G, Sundan A, Waage A, Austgulen R 1993 Tumor necrosis factor, interleukin 1, and interleukin 6 in normal pregnancy. *Am J Obstet Gynecol* 169:397–404
3. Wegmann TG, Guilbert LJ 1992 Immune signalling at the maternal-fetal interface and trophoblastic differentiation. *Dev Comp Immunol* 16:425–430
4. Guilbert L, Robertson SA, Wegmann TG 1993 The trophoblast as an integral component of a macrophage-cytokine network. *Immunol Cell Biol* 71:49–57
5. Wegmann TG, Lin H, Guilbert L, Mosmann TR 1993 Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a T_H2 phenomenon? *Immunol Today* 7:353–356
6. Abbas A, Snijders RJ, Buggins AG, Layton DM, Nicolaidis KH 1993 Fetal plasma tumor necrosis factor concentration in normal pregnancy. *Fetal Diagn Ther* 8:220–223
7. Berry SB, Fine N, Bichalski JA, Cotton DB, Dombrowski MP, Kaplan J 1993 Circulating lymphocyte subsets in second- and third-trimester fetuses: comparison with newborns and adults. *Am J Obstet Gynecol* 167:895–900
8. Chomczynski P, Sacchi N 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156–159
9. Weatherstone KB, Rich EA 1989 Tumor necrosis factor/cachectin and interleukin-1 secretion by cord blood monocytes from premature and term neonates. *Pediatr Res* 25:342–346
10. Lathley JL, Kanangat S, Rouse BT 1994 Differential expression of tumor necrosis factor α and interleukin 1 β compared with interleukin 6 in monocytes from human immunodeficiency virus-positive individuals measured by polymerase chain reaction. *J Acquir Immune Defic Syndr* 7:109–115