Amniotic fluid interleukin 6 in preterm labor. Association with infection.

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J Clin Invest. 1990;85(5):1392-1400. https://doi.org/10.1172/JCI114583.

Research Article

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Amniotic Fluid Interleukin 6 in Preterm Labor

Association with Infection

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Abstract

To evaluate whether IL-6 participates in the host response to intrauterine infection, we studied IL-6 bioactivity and isoforms in amniotic fluid (AF). Two different assays for IL-6 were used: the hepatocyte stimulating factor assay (in Hep3B2 cells) and the SDS-PAGE/immunoblot assay. IL-6 determinations were performed in 205 AF samples. Samples were obtained from patients in the midtrimester of pregnancy (n = 25), at term with no labor (n = 31), at term in active labor (n = 40), and from patients in preterm labor (n = 109). Higher AF IL-6 levels were observed in women in preterm labor with intraamniotic infection than in women in preterm labor without intraamniotic infection (median = 375 ng/ml, range = 30–5000 ng/ml vs. median = 1.5 ng/ml, range = 0–500, respectively, $P < 0.0001$). The 23–25- and 28–30-kD IL-6 species could be readily detected in SDS-PAGE immunoblots performed directly on 10-μl aliquots of AF from patients with intraamniotic infection. Among women in preterm labor with culture-negative AF, those who failed to respond to subsequent tocolytic treatment had higher AF IL-6 concentrations than those who responded to therapy (median = 50 ng/ml vs. median = 1.2 ng/ml, respectively, $P < 0.05$). Only low levels of IL-6 were detected in AF obtained from normal women in the midtrimester and third trimester of pregnancy. Decidual tissue explants obtained from the placentas of women undergoing elective cesarean section at term without labor (n = 11) produced IL-6 in response to bacterial endotoxin. In a pilot study, AF IL-6 was determined in 56 consecutive women admitted with preterm labor. All patients (n = 10) with elevated AF IL-6 (cutoff = 46 ng/ml) delivered a premature neonate. 4 of these 10 patients had positive AF cultures for microorganisms. These studies implicate IL-6 in the host response to intrauterine infection and suggest that evaluation of AF IL-6 levels may have diagnostic and prognostic value in the management of women in preterm labor. (*J. Clin. Invest.* 1990. 85:1392–1400.) **prematurity • parturition • decidua • cytokines • bacterial endotoxins**

Introduction

Prematurity is the leading cause of perinatal mortality and morbidity worldwide (1). A growing body of evidence suggests an association between subclinical intrauterine infection and preterm labor (PTL)[1] (reviewed in reference 2). Recently, we have estimated that at least one of every five premature neonates is born to a mother with an intraamniotic infection (3, 4). The majority of these infections are subclinical and difficult to diagnose (3, 4). The participation of cytokines in the inflammatory process associated with intraamniotic infection is supported by the detection of IL-1 and tumor necrosis factor (TNF) in the amniotic fluid (AF) of women with this condition (5, 6). The purpose of this study was to evaluate the participation of IL-6 in the pathophysiology of premature labor.

IL-6 has been implicated as a major mediator of the host response to infection and tissue damage (7, 8). This cytokine consists of a group of differentially modified phosphoglycoproteins ranging in size from 23 to 30 kD and additional complexes of 43–45 kD (8–13) derived from a single gene located at 7p21 in the human genome (14–16). IL-6 gene expression is induced by several inflammation-associated cytokines (including IL-1, TNF, and interferons), bacterial products, RNA- and DNA-containing viruses, and second messenger agonists (diacylglycerol, cAMP, and Ca^{2+}) that activate any of the three major signal transduction pathways (17–26). Cell types capable of secreting IL-6 in response to stimulation include fibroblasts, monocytes/macrophages, endothelial cells, keratinocytes, and endometrial stromal cells (7–27).

IL-6 has a broad range of biological effects (reviewed in reference 25). It elicits major changes in the biochemical, physiological, and immunological status of the host (e.g., the "acute phase" plasma protein response, activation of T and NK cells, and stimulation of proliferation and immunoglobulin production by B cells). The alterations in plasma protein composition mediated by IL-6 are thought to seal the site of tissue injury and reduce the systemic effects of infection and tissue damage (7, 25). For example, increased concentrations of fibrinogen and other coagulation factors may promote thrombus formation.

The acute phase plasma protein response may be important in the context of intraamniotic infection. Clinical studies have indicated that elevation of maternal serum C-reactive protein (CRP) often precedes the development of clinical chorioamnionitis and the onset of premature labor in women with preterm premature rupture of membranes (28–30). Furthermore, patients in PTL with elevated levels of CRP are more likely to be unresponsive to tocolytic therapy than those with nondetectable CRP (30–32). As IL-6 plays a critical role in the induction of CRP synthesis (33, 34), it was considered likely that this cytokine participated in the host response to intrauterine infection.

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Received for publication 29 June 1989 and in revised form 7 December 1989.

J. Clin. Invest.

0021-9738/90/05/1392/09 $2.00
Volume 85, May 1990, 1392–1400

1. *Abbreviations used in this paper:* ACT, α_1-antichymotrypsin; AF, amniotic fluid; CRP, C-reactive protein; HSF, hepatocyte stimulating factor; PTL, preterm labor; r, recombinant; TL, term labor; TNF, tumor necrosis factor.

IL-6 bioactivity was measured and IL-6 isoforms were characterized in the AF of women with and without intraamniotic infection. Highly elevated AF IL-6 levels were observed in women with PTL and intraamniotic infection who progressed to delivery. AF samples from this group of patients contained the highest levels of IL-6 observed by us in any human body fluid. Indeed, the levels were so high (median = 375 ng/ml; range = 30–5,000 ng/ml) that the 23–25- and 28–30-kD IL-6 species could be readily detected in immunoblots performed directly on 10-μl aliquots of the fluid. Women with PTL unresponsive to tocolysis had a higher median AF concentration of IL-6 than women who responded to tocolysis. These observations were confirmed in a pilot study of 56 consecutive women admitted with PTL. All patients with elevated AF IL-6 (cutoff = 46 ng/ml) subsequently delivered a premature neonate. Furthermore, all patients with culture-positive AF had elevated IL-6 in their AF as defined in this assay. These data suggest that elevated AF IL-6 levels may be of value in the detection of intraamniotic infection and may also provide prognostic information regarding the effectiveness of tocolysis in impeding preterm delivery.

Methods

Patient population and collection of AF. AF was collected from women in four different populations: group 1, women admitted with PTL and intact membranes on whom amniocenteses were performed for microbiological assessment of the amniotic cavity and for fetal lung maturity studies (n = 109); group 2, women in the midtrimester of pregnancy (gestational age: 16–18 wk) who underwent amniocenteses for genetic indications (maternal age > 35 yr) (n = 25); group 3, women in the third trimester (gestational age: 37–40 wk) who had amniocenteses for the assessment of fetal lung maturity before elective cesarean section (n = 31); and group 4, women in spontaneous active labor (cervical dilatation of at least 6 cm) at 38–41 wk (term labor [TL]) from whom AF was obtained by transvaginal amniotomy (n = 40).

To study the relationship between intraamniotic infection, PTL, and AF IL-6 concentrations, a cross-sectional study of patients in group 1 (PTL) was constructed. Patients with PTL were divided into three subgroups according to their response to tocolysis and the results of their AF culture: subgroup 1a, women with PTL and negative AF cultures who were responsive to tocolysis; subgroup 1b, women with PTL and negative AF cultures who were unresponsive to tocolysis and delivered a preterm neonate; and subgroup 1c, women with PTL and intraamniotic infection who delivered a preterm neonate. In addition, we also conducted a pilot study to explore the potential clinical value of AF IL-6 determinations. This cytokine was assayed in the AF of 56 consecutive women admitted with the diagnosis of PTL.

An intraamniotic infection was considered to be present if microbiological studies of AF yielded a positive result (2). PTL was defined as the presence of regular uterine contractions with a frequency of at least two every 10 min for at least 60 min. A β_2-adrenergic drug (ritodrine in most cases) was administered intravenously as the tocolytic agent according to a protocol described elsewhere (35). Amniocentesis was performed before the initiation of therapy. Failure of tocolysis was diagnosed when cervical dilatation progressed beyond 5 cm and/or delivery occurred.

After defining the criteria for entry into the study, AF samples were obtained from the AF bank in the Department of Obstetrics and Gynecology, Yale University School of Medicine (New Haven, CT). This bank consists of aliquots of AF that were not used for clinical testing. AF samples were centrifuged at 400 g for 10 min at 4°C immediately after collection, and the fluid was separated from the cell pellet and stored frozen at −70°C. AF samples analyzed for IL-6 in this study were from consecutive patients admitted to the hospital who fulfilled the criteria of each of the study groups. Informed consent was obtained from all patients following the guidelines of the Yale University Human Investigations Committee.

Microbiological studies. AF in a capped plastic syringe was transported to the microbiology laboratory immediately after collection and plated within 30 min of amniocentesis. AF was cultured for aerobic and anaerobic bacteria as well as for *Mycoplasma hominis* and *Ureaplasma urealyticum,* as described previously (36). AF samples from women in groups 2 and 3 were sterile. AF cultures were not performed on samples obtained by transvaginal amniotomy (group 4). Previous experience has indicated that the results of these cultures do not reflect the microbiological status of the amniotic cavity because contamination with cervical or vaginal flora cannot always be avoided during transvaginal amniotomy (2).

Cultures of decidual tissue explants. Decidual explants were obtained from 11 pregnant women at term who had experienced uncomplicated pregnancies and were scheduled to have elective repeat cesarean sections. None had spontaneous onset of labor before surgery. Care was taken to avoid contact of the placenta with the skin or other potential sources of endotoxin. Immediately after delivery of the placenta, the decidua adherent to the chorion was mechanically stripped with sterile forceps. The explants were placed in ice-cold, pyrogen-free Krebs-Ringer's solution. They were incubated in DME (Gibco Laboratories, Grand Island, NY) with 10% FCS, 100 U/ml of penicillin, streptomycin (final concentration 100 μg/ml), and 4 μM L-glutamine. Explants were ~ 1 cm × 1 cm in size and were incubated in 2 ml of media in 35-mm plastic tissue culture dishes (Corning Glass Works, Corning, NY). Explants were incubated at 37°C in a humidified incubator with 95% O_2 and 5% CO_2 for 20 h. The medium was centrifuged at 200 g for 10 min. Supernatants were removed and frozen immediately at −20°C until the time of the assay.

Hepatocyte stimulating factor (HSF) assay for IL-6 biological activity. The levels of biologically active IL-6 in AF samples were estimated using the HSF assay as previously described in detail (9, 10, 12, 13). Briefly, the human hepatoma cell line Hep3B clone 2, obtained from the American Type Culture Collection (Cat. No. HB8064; Rockville, MD), was grown to near-confluence in 24-well tissue culture plates (Falcon Plastics, Cockeysville, MD). Appropriately diluted AF samples (diluted 1:5–1:250) were added to Hep3B2 cultures (0.5 ml/well) in the presence of excess insulin (8 μg/ml) and dexamethasone (1 μM). After incubation for 20–24 h at 37°C, the cultures were washed with PBS and incubated for an additional 24 h in methionine-free medium containing [^{35}S]methionine (100 μCi/ml; DuPont-New England Nuclear, Boston, MA). The culture medium from each well was then collected, and the amount of labeled α_1-antichymotrypsin (ACT) secreted was quantitated by immunoprecipitation (rabbit antiserum to human ACT was purchased from Calbiochem-Behring Corp., San Diego, CA), SDS-PAGE, autoradiography, and densitometry (Ultroscan XL laser densitometer; LKB Instruments, Inc., Gaithersburg, MD) using procedures described earlier (10, 12, 13). A purified preparation of natural human IL-6 (produced by IL-1α-induced fibroblasts) was used as an internal laboratory standard, and appropriate dilutions of this preparation were also assayed in duplicate in every experiment. The concentration of IL-6 in the laboratory standard (2 μg/ml) was determined by silver-staining of the IL-6 proteins after SDS-PAGE, and the same value (2 μg/ml) was also arrived at independently by comparing this mixture with electrophoretically homogeneous Coomassie blue–stained *Escherichia coli*–derived recombinant (r) IL-6 (8) in immunoblots using rabbit anti–rIL-6 (8). Concentrations of biologically active IL-6 in AF are expressed in nanograms/milliliter by comparison to this laboratory standard.

The biological activity in AF samples observed in this HSF assay was verified to be due to IL-6, based on the ability of a rabbit polyclonal antiserum (1:100 dilution) prepared against purified *E. coli*–derived human IL-6 (8) to completely neutralize the activity. The properties and characteristics of this anti-rIL-6 antiserum have been extensively described previously (8–13, 37, 38). This antiserum specifically blocks IL-6 activity not only in hepatocyte stimulation assays but also

in B cell growth and differentiation assays, hybridoma growth assays, and T cell activation assays. We have verified that IL-1α at concentrations up to 50 ng/ml, IL-1β at 2 ng/ml, TNF at concentrations up to 1 μg/ml, and bacterial LPS up to 5 μg/ml do not affect ACT synthesis in the Hep3B2 cells used (8, 10, and data not shown). Nevertheless, AF samples in group 1 (PTL) were also assayed in the presence of excess neutralizing antibodies to both IL-1α and IL-1β (2 μg/ml each), because some of these AF samples were previously found to contain immunoreactive IL-1β (up to a maximum of 2–4 ng/ml; Romero, R., unpublished observations). The murine neutralizing monoclonal antibodies to human IL-1α or IL-1β were kindly provided by Dr. A. C. Allison (Syntex Research, Palo Alto, CA). At 1 μg/ml, both antibodies block 1 ng/ml of IL-1α or IL-1β in the thymocyte comitogenic assay and the fibroblast proliferation assay (39). An anti-TNF monoclonal antibody was obtained from the Suntory Institute for Biomedical Research (Osaka, Japan); 1 μg/ml of this antibody neutralizes 2 ng/ml of rTNF activity in the L929 cytotoxicity assay.

In summary, the HSF assay for biologically active IL-6 consists of two parts: (*a*) estimation of the enhancement of ACT synthesis in Hep3B2 cells, and (*b*) inhibition of this enhancement by anti-rIL-6. A major advantage of this assay is that it is not affected by the presence of bacterial products in the test samples.

Immunoblot assay for IL-6 proteins. AF samples from women with PTL and intact membranes (group 1) were also assayed for IL-6 content using an immunoblot procedure (8–10, 12, 13). 10-μl aliquots of AF were electrophoresed through SDS-PAGE (17.5%) under reducing and denaturing conditions, electroblotted onto nitrocellulose paper, and probed using rabbit anti-rIL-6 (8–13) and the ABC Elite Vectastain kit (Vector Laboratories, Inc., Burlingame, CA). Each blot also contained appropriate dilutions of the laboratory standard for natural IL-6. As controls, additional blots were probed using rabbit preimmune serum, serum from an unrelated rabbit, or the first antibody omitted from the reaction.

Statistical analysis. Comparisons of IL-6 estimates in AF samples from different groups were conducted using a Kruskal-Wallis one-way analysis of variance. The Dunn test was used for post-hoc multiple comparisons among groups (True Epistat; Epistat Services, Richardson, TX).

Results

We have evaluated IL-6 bioactivity in AF from women at midtrimester, at term not in labor, at term in active spontaneous labor, and from patients in PTL with and without intraamniotic infection. We shall focus on the results of AF IL-6 from women with PTL because these patients presented with intact membranes, and, therefore, a positive AF culture is a reliable indicator of microbial invasion of the amniotic cavity.

IL-6 in AF in PTL. Fig. 1 summarizes a representative IL-6/HSF assay carried out on AF samples from women in PTL. A 1:5 dilution of AF from six patients in each of the three subgroups 1a, 1b, and 1c (as defined in Methods), is illustrated. As can be seen, the IL-6/HSF activity of AF samples from women in PTL who were responsive to tocolysis and who were AF culture-negative (subgroup 1a) is the lowest. That of AF samples of women in PTL who were not responsive to tocolysis and who were AF culture-positive (subgroup 1c) is the highest (also see Figs. 3 and 4). It is striking that all of the AF samples in subgroup 1c (Figs. 1 *C*, 3, and 4) were strongly positive for IL-6/HSF activity.

That the biological activity observed in this IL-6/HSF assay in these and other AF samples was due specifically to IL-6 was verified by a separate set of experiments similar to those summarized in Fig. 2. Appropriate dilutions of AF (three each from Fig. 1, *B* and *C* and three from a separate group with

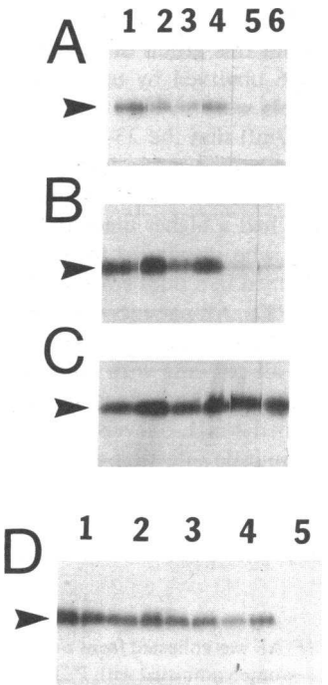

Figure 1. IL-6/HSF bioactivity in the AF of women with PTL. AF diluted 1:5 (500 μl final volume) from women in PTL was assayed for its ability to enhance synthesis and secretion of [^{35}S]methionine-labeled ACT in Hep3B2 cultures. The figure is a composite of data in one autoradiogram and shows the stimulation of ACT synthesis (*arrowheads*) by AF samples from six different patients in each of the following three subgroups: *A*, AF culture-negative and PTL responsive to tocolysis (subgroup 1a); *B*, AF culture-negative and PTL not responsive to tocolysis (subgroup 1b); and *C*, AF culture-positive and PTL not responsive to tocolysis (subgroup 1c). For comparison, *D* illustrates ACT stimulation by the natural IL-6 standard preparation at 10, 5, 2, 1, and 0 ng/ml in duplicate assays in sets 1, 2, 3, 4, and 5, respectively.

PTL and intraamniotic infection) were mixed with a 1:100 dilution of the anti-rIL-6 antibody (lanes *a*), and the residual HSF activity was assayed. The data shown in Fig. 2 clearly demonstrate that this anti-rIL-6 antibody was able to strongly inhibit the HSF activity observed. Additionally, we have veri-

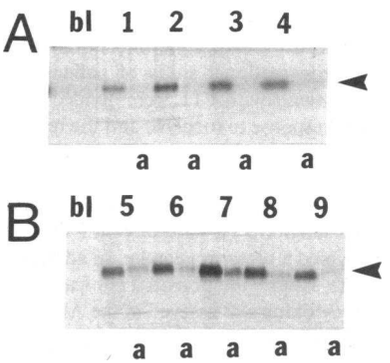

Figure 2. Neutralization of AF IL-6/HSF bioactivity by anti-rIL-6 antiserum. The figure illustrates composite autoradiograms from two different experiments (*A* and *B*) in which the ability of appropriately diluted (1:20–1:100) AF samples (500 μl final volume) to stimulate ACT synthesis (*arrowheads*) in Hep3B2 cultures was assayed after incubation with rabbit anti-rIL-6 antiserum at 1:100 dilution (lanes *a*). Three AF samples from each of the following three groups were evaluated: A2, B5, B6, AF culture-negative, and PTL not responsive to tocolysis (subgroup 1b; these three samples are from among the six illustrated in Fig. 1 *B*); A1, A3, B7, AF culture-positive, and PTL not responsive to tocolysis (subgroup 1c; these three samples are from among the six illustrated in Fig. 1 *C*); A4, B8, B9 correspond to a separate set of samples from women with PTL and infection. In additional experiments, control unrelated rabbit serum at 1:100 dilution had little effect on the stimulation of ACT synthesis (not shown). Residual HSF activity seen in some of the lanes (particularly *7a*), even in the presence of anti-rIL-6 at 1:100 dilution, is consistent with the very high concentrations of IL-6 in these samples (200–5,000 ng/ml as subsequently verified by immunoblot assays; see Fig. 3). *bl*, ACT synthesis in unstimulated control Hep3B2 cultures.

fied in similar neutralization experiments that the following reagents have little or no effect on the HSF activity observed in AF samples: (*a*) preimmune or unrelated rabbit serum, (*b*) neutralizing anti-TNF antibody, and (*c*) a combination of the neutralizing anti-IL-1α and anti-IL-1β antibodies. We thus conclude that the observed HSF biological activity in AF samples can be ascribed to IL-6.

To substantiate this conclusion further, we have evaluated the IL-6 content of AF samples from women in PTL by immunoblot analysis. This procedure allows characterization of the IL-6 species present in AF. Fig. 3 illustrates immunoblot data obtained from 10 samples each from subgroups 1a and 1c. It is clear that AF samples in subgroup 1c (PTL, not responsive to tocolysis and AF culture-positive) contain IL-6 at such high concentrations that the 23–25- and 28–30-kD species of this cytokine can be readily detected using only a 10-μl aliquot in this assay. The data in Fig. 3 are consistent with those in Fig. 1 in that the lowest IL-6 concentrations are seen in AF samples in subgroup 1a, and the highest levels in subgroup 1c. Immunoblot assays on the AF samples in group 1 were consistent with data obtained using the HSF bioassay (data not shown). Strikingly, all of the samples in subgroup 1c are strongly positive for IL-6 in both the HSF and immunoblot assays (also see below).

The immunoblot data in Fig. 3 provide the first description of IL-6 species present in human AF. These data show that differentially modified IL-6 species (the 23–25-kD species are *O*-glycosylated; the 28–30-kD species are *O*- and *N*-glycosylated [8, 10, 25]) are indeed present in human body fluids. These immunoblot assays reveal that IL-6 isoforms are present in AF at concentrations as high as 1–5 μg/ml.

The concentrations of IL-6 in all the AF samples from women in PTL (group 1) were estimated using the HSF assay. AF samples were diluted as appropriate (up to 1:250) to obtain a stimulation in ACT synthesis that would be within the log-linear range of the assay. Furthermore, strongly positive samples (in groups 1b and 1c) were reassayed in the presence of excess neutralizing antibody to both IL-1α and IL-1β. Fig. 4 *a* summarizes the estimates of IL-6 content in all of the AF samples in the group with PTL. Patients with intraamniotic infection had significantly greater concentrations of AF IL-6 than women without intraamniotic infection regardless of their response to tocolysis (median = 375 ng/ml, range = 30–5,000 ng/ml vs. median = 1.5 ng/ml, range = 0–500, respectively, $P < 0.0001$). Patients without intraamniotic infection but refractory to tocolytic therapy had higher median concentrations of AF IL-6 than those women without intraamniotic infection who responded to tocolysis ($P < 0.05$) (Fig. 4 *b*).

The clinical picture of several outliers is noteworthy. One patient with PTL responsive to tocolysis had an AF IL-6 level of 125 ng/ml. This patient presented with PTL and a temperature of 100°F. Her white blood cell count was 12,200 with a differential count of 81% segmented neutrophils and 5% monocytes. Although AF and urine cultures were negative, it is possible that this patient had a self-limiting inflammatory reaction that could not be diagnosed. She delivered at term 16 d later.

Two women in the group of patients with PTL who were unresponsive to tocolysis (group 1b) had AF IL-6 concentrations of 500 ng/ml. AF from one of these patients stained positive for gram-positive cocci and gram-negative rods, but the culture grew only *Corynebacterium* species. Isolation of this microorganism is generally interpreted as consistent with a skin contaminant. This interpretation may have been erroneous. The presence of elevated IL-6 suggests that there was an ongoing inflammatory reaction in the amniotic cavity. The second patient was clinically suspected to have chorioamnionitis because of the association of fever and PTL. Although the gram stain of AF was negative for bacteria, and the culture was negative, histopathologic examination of the placenta showed intense chorioamnionitis. Therefore, this patient may have had an intraamniotic infection that escaped detection using current microbiologic techniques, an infection limited to the extraamniotic membranes, or an inflammatory reaction of a noninfectious etiology. In any case, the high AF IL-6 levels appear to be indicative of an ongoing inflammatory process.

Elevated AF IL-6 levels are associated with intraamniotic infection by a variety of microorganisms including gram-positive and gram-negative species and *Ureaplasma urealyticum* (see Table I). These data strongly suggest that elevated AF IL-6 levels are indicative of an inflammatory reaction associated with infection regardless of the specific microorganism involved.

IL-6 in the AF from spontaneous TL. We extended our studies of AF IL-6 levels during parturition to a group of patients in spontaneous TL (group 4). As controls, we studied AF IL-6 levels in patients in midtrimester (group 2) and in women at term but not in labor (group 3). Fig. 5 summarizes the data obtained. AF from women in the midtrimester and third trimester (at term) of pregnancy contained detectable but low levels of IL-6 (median 10 ng/ml in group 2 and 13 ng/ml in group 3). However, AF IL-6 levels were higher in women in spontaneous labor at term (group 4) than in women who were

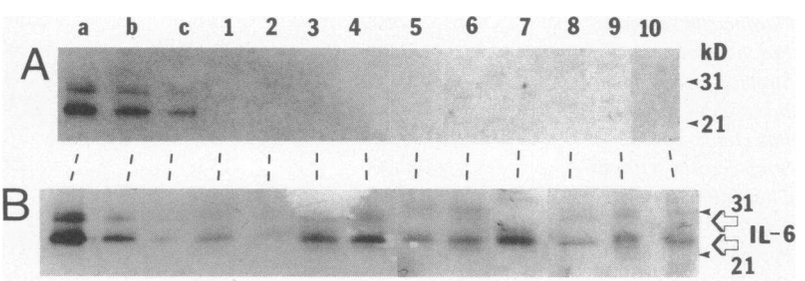

Figure 3. Multiple forms of IL-6 proteins in AF in PTL. This composite figure illustrates SDS-PAGE/immunoblot assays for IL-6 carried out using 10-μl AF aliquots (undiluted final volume) from 10 patients in each of the following two groups: *A*, AF culture-negative and PTL responsive to tocolysis (subgroup 1a, two of the samples are from among the six illustrated in Fig. 1 *A*); *B*, AF culture-positive and PTL not responsive to tocolysis (subgroup 1c, four of the samples are from among the six illustrated in Fig. 1 *C*). Lanes *a*, *b*, and *c* in each panel illustrate immunoblot assays using the natural IL-6 standard preparation at 4, 2, and 1 ng of antigen per lane in *A* and at 10, 2, and 0.4 ng per lane in *B*. In additional immunoblot analyses of AF samples, unrelated rabbit serum or the second anti-rabbit antibody by itself did not react with the 23–25- and 28–30-kD anti-rIL-6-immunoreactive proteins (data not shown).

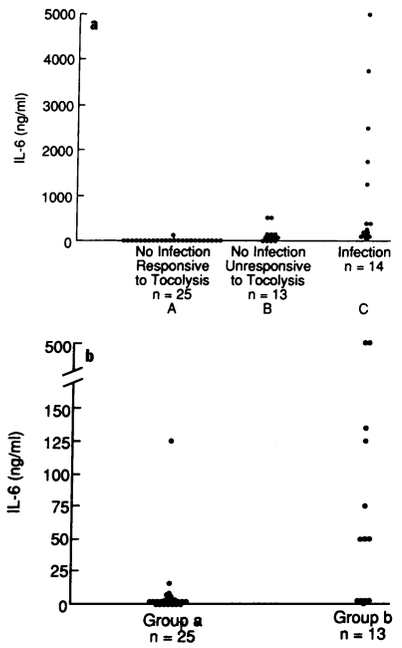

Figure 4. AF IL-6 levels in women with PTL. *a,* Scatter diagram illustrating AF IL-6 levels in three different subgroups of patients: A, AF culture-negative and responsive to tocolysis (subgroup 1a, $n = 25$; median IL-6 = 1.2 ng/ml, range = 0–125 ng/ml); B, AF culture-negative and not responsive to tocolysis (subgroup 1b, $n = 13$; median IL-6 = 50 ng/ml, range = 0–500 ng/ml); C, AF culture-positive and not responsive to tocolysis (subgroup 1c, $n = 15$; median IL-6 = 375 ng/ml, range = 30–5,000 ng/ml). Kruskal-Wallis $H = 30.425$; $P = 0.0000002$; A compared with B, $P < 0.05$; B compared with C, $P < 0.05$; C compared with A, $P < 0.05$ (Dunn's test). *b,* Replot of data for subgroups A and B shown in *a.*

not in labor at term (group 3) (median = 19 ng/ml, range = 4–500 for women in active labor vs. median = 13 ng/ml, range = 3.5–60 ng/ml; $P < 0.01$). The major difference between these two groups can be attributed to several patients in active labor who had marked elevations of AF IL-6 levels. A subclinical intraamniotic infection is the most likely explanation. Indeed, previous histopathologic studies indicate that up to 10% of normal term placentas have inflammatory lesions consistent with the presence of an intraamniotic infection (32).

Bacterial LPS induces IL-6 production by decidual tissue explants. We have tested whether decidual tissue explants from normal placentas can be induced to secrete IL-6 by bacterial LPS. Fig. 6 summarizes an evaluation of IL-6 bioactivity in the medium of uninduced and LPS-induced decidual tissue explant cultures prepared from the placentas of 11 different women. Tissue from each individual placenta was cultured in the absence (lanes *u*) or the presence (lanes *l*) of bacterial LPS (25 ng/ml) for 24 h. Fig. 6 illustrates that several of the decidual tissue explants spontaneously secrete significant amounts of IL-6 and that bacterial endotoxin increased IL-6 production by all explants. In additional assays, we have verified that anti-rIL-6 strongly inhibits the IL-6 activity in LPS-induced samples illustrated in Fig. 6. These data are consistent with the hypothesis that bacterial products can stimulate tissues in the maternal–fetal interface to produce IL-6.

Clinical value of AF IL-6 measurements. We designed a pilot study to explore the potential value of routine IL-6 measurements in clinical practice. AF samples from 56 consecutive women with the clinical diagnosis of PTL were tested for IL-6 content. Of these patients, 25 subsequently delivered a preterm neonate and 31 delivered at term. IL-6 content was assayed in this pilot study using the HSF assay, in which the stimulation of [^{35}S]methionine-labeled ACT was monitored by immunoprecipitation, PAGE, and autoradiography. Visual inspection of this autoradiogram allowed us to readily identify 10 AF samples that had clearly elevated IL-6 activity. Subsequent quantitation revealed that this subgroup of 10 fluids had AF IL-6 levels that were equal to or greater than 46 ng/ml. We then tabulated the clinical outcome in these 10 patients; all these patients delivered preterm neonates. Clinical information, microbiological results, and placental pathology of these patients are displayed in Table II. It is noteworthy that the four patients with positive AF cultures for microorganisms also had elevated AF IL-6. Furthermore, several of the patients who had elevated AF IL-6 but negative AF cultures had histopathologic evidence of chorioamnionitis.

Table I. Microbiological Data and IL-6 in Women with PTL and Intact Membranes

Patient identification No.	Gram stain	Gestational age	Organism	Colony count	Amniocentesis to delivery interval	AF IL-6
				cfu/ml		*ng/ml*
1	+	25	*Streptococcus viridans*	>100,000	4 h	30
2	−	27	*Klebsiella pneumonia*	>100,000	45 min	100
3	−	25	Group B *Streptococcus*	>100,000	6 h	1,750
4	+	25.5	Group B *Streptococcus*	>100,000	—	5,000
5	+	22	*Gardnerella vaginalis*	500	13 h	5,000
6	−	26	*Fusobacterium nucleatum*	2,000	11 h	200
7	+	28	*Streptococcus viridans*	>100,000	6.5 h	100
8	+	24	*Bacteroides* species	>100,000	8 h	3,750
9	+	24	*Bacteroides* species	>100,000	4 h	375
10	+	29	*Streptococcus viridans*	>100,000	13 h	250
11	+	26	*Clostridium* species *Capnophage* species	>100,000	8 h	1,250
12	+	34	*Peptostreptococcus*	>100,000	5 h	375
13	+	25	*Candida tropicalis*	—	19 h	100
14	−	27	Mixed anaerobic flora	—	46 h	200
15	+	26	*Fusobacterium nucleatum*	500	12 d	2,500

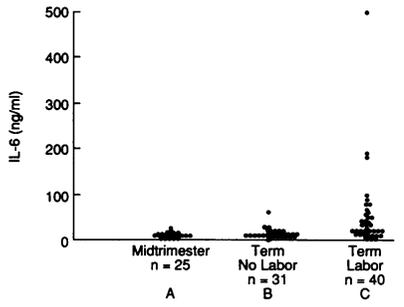

Figure 5. AF IL-6 levels in women in midtrimester and at term. Scatter diagram illustrating AF IL-6 levels in three different groups of patients: A, midtrimester (group 2, $n = 25$; median IL-6 = 10 ng/ml, range = 2–27 ng/ml); B, at term but not in labor (group 3, $n = 31$; median IL-6 = 13, range = 0–60 ng/ml); and C, at term and in spontaneous labor (group 4, $n = 40$; median IL-6 = 19.5 ng/ml, range = 4–500 ng/ml). Kruskal-Wallis H = 23.35; $P = 0.0000085$; A compared with B, $P > 0.05$; B compared with C, $P < 0.05$; C compared with A, $P < 0.05$ (Dunn's test).

Discussion

This is the first report to describe the presence of IL-6 in AF. We describe a dramatic increase in the AF IL-6 levels in women with intrauterine infection and PTL. The data obtained suggest that AF IL-6 levels may be of diagnostic and prognostic value in the management of PTL.

Biologically active IL-6 was detected in AF using the HSF assay in Hep3B2 cells; this bioactivity was confirmed to be due to IL-6 per se by its neutralization with an anti-rIL-6 antiserum. Although the HSF assay is not as sensitive as other bioassays available for IL-6 (e.g., the hybridoma growth factor assay) (25), it is robust and insensitive to the presence of bacterial products in the body fluid tested. Immunoblot analyses confirmed the presence of the 23–25- and 28–30-kD IL-6 species in AF. This extends previous descriptions of the heterogeneity of IL-6 species in human serum/plasma, cerebrospinal fluid, and synovial fluid (10, 12, 13, 25) to the AF. IL-6 heterogeneity is, thus, a very general feature of this cytokine in human body fluids and is due to *N*- and *O*-glycosylation and differential phosphorylation of the 23–30-kD IL-6 species (8, 9, 25). The 23–25-kD IL-6 species are *O*-glycosylated, whereas the 28–30-kD IL-6 species are both *O*- and *N*-glycosylated. Additionally, higher molecular mass immunoreactive complexes (43–45-kD) have been reported in serum/plasma and in synovial fluid (10–13). In cell culture, induced human fibroblasts, monocytes, endothelial cells, keratinocytes, and endometrial stromal cells have all been shown to secrete multiple differentially modified IL-6 species of molecular mass 23–30 kD in cell culture (8, 9, 11, 25). The biological consequences of this heterogeneity are unclear.

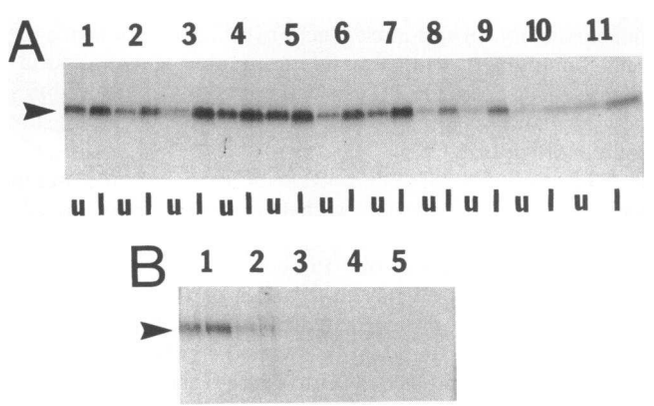

Figure 6. Production of IL-6 by decidual tissue explants in response to LPS. Decidual tissue explants from 11 placentas from women undergoing elective cesarean sections without spontaneous labor were cultured overnight in the absence (*u*) or presence of LPS (25 ng/ml) (*l*), and the accumulation of IL-6 bioactivity in the culture medium was monitored (*A*). *B* illustrates the stimulation of ACT synthesis in duplicate cultures by the natural IL-6 standard preparation used at 10, 2.5, 1, 0.4, and 0 ng/ml in this experiment (sets 1–5).

A major finding in the present study is that the AF of women with PTL and intraamniotic infection contained very high levels of IL-6. All of the AF samples from women with PTL and infection were strongly positive for IL-6. This indicates that IL-6 is a participant in the host response to intraamniotic infection. Microbiologic data from these patients demonstrate that elevated AF IL-6 levels are observed in women with intraamniotic infections due to a wide variety of organisms including gram-negative and gram-positive bacterial species.

It should be stressed that PTL leading to preterm delivery in the absence of detectable infection was also associated with elevations in AF IL-6 levels, although of a lesser magnitude than that observed in cases of intraamniotic infection. Several possible explanations for this observation may be considered. First, this subgroup of women may have had an intrauterine inflammatory reaction unrelated to intraamniotic infection (i.e., an extraamniotic infection or a noninfectious inflammatory process). Second, an elevation of IL-6 may be associated with the physiologic process of parturition. Third, an intraamniotic infection may have escaped detection using standard microbiological techniques. This latter possibility is a likely explanation for two specific cases with elevated AF IL-6 concentrations (500 ng/ml). The first case had a positive gram stain of AF for bacteria, but the AF grew *Corynebacterium* species, which are considered a skin contaminant. The second case had clinical and histopathologic evidence of chorioamnionitis, but no bacteria could be recovered. Further studies are required to clarify these issues.

A perennial clinical problem is the interpretation of microbiological cultures of biological fluids in which the initial gram stain and culture yield conflicting results. A potential role for IL-6 in clinical practice could be to help identify between sample contamination or culture failure. For example, a positive gram stain of AF with elevated IL-6 levels is more likely to be due to a true intraamniotic infection even with a negative AF culture. Additionally, in one of the cases studied by us, it is unclear whether the *Corynebacterium* isolated was a skin contaminant introduced into the sample during the amniocentesis procedure or a true pathogen. The identification of microorganisms in gram stains coupled with a high level of IL-6 in AF suggests a pathogenic role for microorganisms. This concept broadens the potential clinical utility of cytokine analysis of biological fluids.

In the context of PTL, it is of considerable importance that women who went on to deliver a premature neonate had elevated levels of AF IL-6, regardless of the presence or absence of documented infection. This observation may have important clinical applications in obstetrics. It would seem that a high AF IL-6 level may identify a group of patients who would not benefit from tocolysis. Tocolytic therapy is associated with

Table II. Clinical Data of Women with Elevated AF IL-6 (from a Pilot Study of 56 Consecutive Patients Admitted with PTL)

Serial	Gestational age	Gram stain	AF culture	IL-6	Preterm delivery	Placental chorioamnionitis	Amniocentesis to delivery interval	Comment
1	26	+	*Streptococcus agalactae*	+	+	+	8 h	
2	23	−	*Mycoplasma hominis*	+	+	+	1 wk	
3	25	+	Mixed flora	+	+	+	1 d	
4	33	−	*Ureaplasma urealyticum*	+	+	NA*	5 d	AIDS
5	26	−	Negative	+	+	NA	1 d	Patient had been previously treated with ampicillin.
6	35	−	Negative	+	+	NA	24 h	
7	23	−	Negative	+	+	NA	12 wk	
8	31	−	Negative	+	+	+	3 d	
9	25	−	Negative	+	+	+	34 h	Patient had been previously treated. The neonate died of group B *Streptococcus* sepsis.
10	30	−	Negative	+	+	+	3 wk	

* NA = not available.

significant side effects for both mother and fetus (32, 40). Our findings (also see below) justify additional larger prospective studies to determine whether evaluation of AF IL-6 levels can help identify patients in PTL who will progress to delivery despite tocolytic treatment.

The association between AF IL-6 levels and parturition was also evaluated at term. We observed that the median concentration of AF IL-6 was higher in women in spontaneous labor at term than in women at term who were not in labor. These observations are similar to those reported by us earlier for AF IL-1 bioactivity (5). Inspection of the IL-1 and IL-6 data sets reveals that this difference is due to a subset of patients in active labor who had elevations of both IL-1 and IL-6 in their AF samples. It is possible that this subset consists of patients with subclinical intraamniotic infection or chorioamnionitis. Because the present study did not include histopathologic examination of placentas from women delivering at term, we cannot directly address this question. Nevertheless, our observations provide a basis for constructing a prospective study to explore this question. A limitation of our study is that the method used to collect AF in women in active labor at term was different from that used to retrieve fluid from women at term not in labor (transvaginal amniotomy vs. transabdominal amniocentesis).

After obtaining data that strongly linked elevated AF IL-6 levels to, first, intraamniotic infection and, second, parturition, we designed a pilot study to evaluate the clinical value of AF IL-6 measurements in women with PTL. The most striking observation was that all patients with elevated AF IL-6 went on to deliver a premature neonate. Four of these patients had positive AF cultures, implicating an intraamniotic infection as the etiologic factor responsible for preterm delivery and also for an elevation of AF IL-6. On the other hand, a demonstrable intraamniotic infection (defined as a positive AF culture for microorganisms) was absent in the remaining six patients with detectable AF IL-6. However, histological signs of chorioamnionitis were demonstrated in three of the three cases in which placentas were available for examination. This suggests that an intrauterine infection was present but may have eluded detection with microbiological techniques. This is probably the case in two patients who had been treated with antibiotics before amniocentesis. Antibiotic treatment may have hampered our ability to recover microorganisms but not our ability to detect IL-6 as an index of the host response to infection.

IL-6 in the AF is likely to be of both maternal and fetal origin. We have recently shown that freshly explanted endometrial stromal cells, a normal component of the maternal decidua, are capable of producing IL-6 (27). Similarly, keratinocytes, a normal component of AF due to fetal desquamation, are also capable of producing IL-6 (26). It is likely that inflamed tissues within the uterus, containing a variety of different cell types (macrophages, endothelial cells, and fibroblasts) produce large amounts of IL-6. The observation that decidual tissue explants, which contain many of these cell types, can be induced by LPS to secrete IL-6 is consistent with this possibility. In situ nucleic acid hybridization analyses for cells containing IL-6 mRNA and immunohistochemical localization of IL-6 protein (41) in tissue sections are procedures now available to directly address questions concerning the sources of AF IL-6.

The production of IL-6 is a general feature of processes in which the integrity of a tissue is challenged. Normal pregnancy ending in spontaneous labor calls forth major changes in host biochemistry; intraamniotic infection greatly adds to this stress. Parturition itself may be viewed as part of a repertoire of host defense mechanisms elicited by intraamniotic infection. The production of abundant IL-6 would clearly contribute to the production of protective acute-phase plasma proteins and the activation of immune mechanisms that would help limit tissue damage. IL-6 production at the maternal–fetal interface may restrict tissue damage to the intrauterine compartment and protect the mother from the systemic consequences of disseminated infection. Likewise, the fetus may produce IL-6 in response to localized bacterial invasion (i.e., after aspiration of infected AF), leading to a protective acute phase plasma protein response in the fetus. This interpretation is compatible

with the observation that infected neonates with elevated CRP have higher survival rates than those with nondetectable serum CRP (42, 43).

The present study identifies AF IL-6 as a marker of PTL. A larger prospective study will be necessary to further substantiate the value of AF IL-6 as a marker cytokine in clinical practice. From a biological point of view, we propose that various cytokines may contribute to different aspects of the host response to intrauterine infection. IL-1 and TNF, which strongly stimulate prostaglandin biosynthesis by intrauterine tissues (6, 44), may signal the onset of parturition and also strongly upregulate IL-6 production. IL-6, in turn, may orchestrate biochemical, immunological, and physiological changes that contribute to maternal and fetal survival.

Acknowledgments

We thank Mr. Ralph Zinner for excellent technical assistance.

This research was supported by grants from the Walter Scott Foundation for Medical Research, the National Institutes of Health (AI-16262), a Physician-Scientist Award from the National Institutes of Health (to Dr. Romero), and a contract from the National Foundation for Cancer Research.

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