OBSTETRICS

Amniotic fluid infection, inflammation, and colonization in preterm labor with intact membranes

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OBJECTIVE: The purpose of this study was to compare intraamniotic inflammation vs microbial invasion of the amniotic cavity (MIAC) as predictors of adverse outcome in preterm labor with intact membranes.

STUDY DESIGN: Interleukin-6 (IL-6) was measured in prospectively collected amniotic fluid from 305 women with preterm labor. MIAC was defined by amniotic fluid culture and/or detection of microbial 16S ribosomal DNA. Cases were categorized into 5 groups: infection (MIAC; IL-6, $\geq$11.3 ng/mL); severe inflammation (no MIAC; IL-6, $\geq$11.3 ng/mL); mild inflammation (no MIAC; IL-6, 2.6-11.2 ng/mL); colonization (MIAC; IL-6, $<$2.6 ng/mL); negative (no MIAC; IL-6, $<$2.6 ng/mL).

RESULTS: The infection (n = 27) and severe inflammation (n = 36) groups had similar latency (median, $<$1 day and 2 days, respectively) and similar rates of composite perinatal morbidity and mortality (81% and 72%, respectively). The colonization (n = 4) and negative (n = 195) groups had similar outcomes (median latency, 23.5 and 25 days; composite morbidity and mortality rates, 21% and 25%, respectively). The mild inflammation (n = 47) groups had outcomes that were intermediate to the severe inflammation and negative groups (median latency, 7 days; composite morbidity and mortality rates, 53%). In logistic regression adjusting for gestational age at enrollment, IL-6 $\geq$11.3 and 2.6-11.2 ng/mL, but not MIAC, were associated significantly with composite morbidity and mortality rates (odds ratio [OR], 4.9; 95% confidence interval [CI], 2.2—11.2, OR, 3.1; 95% CI, 1.5—6.4, and OR, 1.8; 95% CI, 0.6—5.5, respectively).

CONCLUSION: We confirmed previous reports that intraamniotic inflammation is associated with adverse perinatal outcomes whether or not intraamniotic microbes are detected. Colonization without inflammation appears relatively benign. Intraamniotic inflammation is not simply present or absent but also has degrees of severity that correlate with adverse outcomes. We propose the designation amniotic inflammatory response syndrome to denote the adverse outcomes that are associated with intraamniotic inflammation.

Key words: chorioamnionitis, intraamniotic infection, intraamniotic inflammation, microbial invasion of the amniotic cavity, morbidity, preterm birth, preterm labor

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Intrauterine infection and inflammation are well-documented causes of preterm labor with intact fetal membranes, especially at very early gestational ages.[1] Cultures for microorganisms in amniotic fluid demonstrate microbial invasion of the amniotic cavity (MIAC) in 20-60% of women with preterm labor at <28 weeks of gestation and 10-25% at 28-32 weeks of gestation.[2-4] Culture-proven MIAC is associated with short latency to delivery and high rates of perinatal morbidity and mortality.[2-5]

Even with culture-negative amniotic fluid, however, women in preterm labor often have intraamniotic inflammation, which is evidenced by elevated amniotic fluid levels of inflammatory markers such as interleukin-6 (IL-6),[3,5-11] other proinflammatory cytokines and chemokines,[6,10-13] tumor necrosis factor alpha,[4,6,10,14] or matrix metalloproteinase-8 (MMP-8).[15-17] Whether or not the amniotic fluid culture is positive, intraamniotic inflammation is associated with short latency[3-5,7,9,12-18] and high rates of perinatal morbidity and mortality.[3-5,14,17,18]

One explanation for the morbidity that is associated with culture-negative intraamniotic inflammation is that many cases actually have MIAC but that the amniotic fluid cultures are falsely negative. Using polymerase chain reaction (PCR) amplification, several groups have demonstrated prokaryotic 16S subunit ribosomal RNA or the DNA coding for it (rDNA) in amniotic fluid in many culture-negative preterm labor cases.[19-26] The microbes that are identified by 16S PCR techniques are often facultative organisms that are difficult to culture with standard techniques. Preterm labor cases with 16S PCR-proven MIAC have similar outcomes to cases with culture-proven MIAC,[19-21,23-25] which suggests true infection and not simply detection of nonviable microbial degradation products.

In principle, MIAC and the intraamniotic inflammatory response are distinct entities. In the simplest model, each of them can be either present or absent; therefore, states of amniotic fluid are possible: (1) infection (MIAC and inflammatory response both present), (2) inflammation (inflammatory response present, MIAC absent); (3) colonization (MIAC present, inflammatory response absent); (4) negative (both absent).

Moreover, the inflammatory response is not simply present or absent but is a continuum. A recent report suggested that clinical outcomes correlated with gradations in inflammatory response that varied from "no" to "minimal" to "severe," with categories defined by the number of biomarkers that are present in amniotic fluid.[27] To our knowledge, there has been no previous report that has investigated whether the severity of outcomes might be graded similarly based on the concentration of a single inflammatory marker.

The aims of the present investigation were to compare the outcomes of preterm labor in women with intraamniotic infection, inflammation, or colonization and to examine whether the outcomes are related to the severity of the inflammatory response as defined by intraamniotic IL-6 levels.

Materials and Methods

This report involved a subset of subjects from a larger multicenter study, the goal of which was to develop a noninvasive test to screen for intraamniotic infection based on cervicovaginal proteins. The protocol was approved by the local institutional review board at each participating site.

Inclusion/exclusion criteria

We included consenting women who were at least 18 years old with singleton pregnancies at 15.0-36.9 weeks of gestation in spontaneous preterm labor with intact fetal membranes and who underwent amniocentesis to evaluate for intraamniotic infection and to measure amniotic fluid IL-6. *Preterm labor* was defined as regular uterine contractions plus at least 1 of the following: cervical dilation ≥2 cm; cervical length by transvaginal sonography ≤30 mm; or a positive cervicovaginal fetal fibronectin test. The protocol required cervical length by transvaginal sonography or fetal fibronectin test only if cervical dilation was <2 cm. Each of the participating sites was a tertiary perinatal center where amniocentesis was offered routinely to women with preterm labor, although several centers restricted the procedure to <34 weeks of gestation. Exclusion criteria were ruptured membranes, major fetal anomaly, fetal aneuploidy, or a medical indication for preterm birth.

TABLE 1
Number of subjects per site by gestational age

Site no.	Gestational age, wk <30	30-33.9	≥34	Total
1	7	9	4	20
3	9	5	0	14
4	14	13	0	27
5	15	4	0	19
6	3	4	0	7
7	2	2	1	5
8	21	7	3	31
9	1	0	0	1
10	15	40	26	81
11	26	10	9	45
12	11	12	8	31
13	2	2	2	6
14	4	5	1	10
16	0	1	0	1
19	0	1	1	2
20	3	1	1	5
Total	133	116	56	305

Combs. Inflammation, infection, preterm labor. Am J Obstet Gynecol 2014.

Specimens

Amniotic fluid was obtained by transabdominal amniocentesis with the use of sonographic guidance and antiseptic skin preparation. A 5-mL aliquot was sent to the local hospital laboratory for assessment of glucose concentration, white blood cell count (WBC), Gram stain, and aerobic and anaerobic culture, which included genital mycoplasmas at some laboratories. A 10-mL aliquot of amniotic fluid was frozen at −80°C and

TABLE 2
Findings in cases with microbial invasion of amniotic cavity

Group classification	Organism identified	16S-ribosomal DNA result	Culture result	Amniotic fluid interleukin-6, ng/mL
Infection	*Bacteroides ureolyticus*	Positive	Positive	206
	Sneathia sanguinegens	Positive	Negative	
	Beta-lactamase positive bacterium	Negative	Positive	
Infection	*Sneathia sanguinegens*	Positive	Negative	199
	Unknown bacterium	Negative	Positive	
Infection	*Fusobacterium nucleatum*	Positive	Positive	166
Infection	*Gardnerella vaginalis*	Positive	Negative	161
	Diphtheroids	Negative	Positive	
	Mobiluncus sp	Negative	Positive	
Infection	*Haemophilus influenzae*	Positive	Positive	147
	Beta-lactamase positive bacterium	Negative	Positive	
Infection	*Fusobacterium nucleatum*	Positive	Positive	106
Infection	*Fusobacterium nucleatum*	Positive	Negative	102
Infection	*Bacteroides ureolyticus*	Positive	Positive	
	Sneathia sanguinegens	Positive	Negative	
	Gardnerella vaginalis	Negative	Positive	
	Acinomyces sp	Negative	Positive	
	Peptostreptococcus sp	Negative	Positive	
	Ureaplasma urealyticum	Negative	Positive	
	Mycoplasma hominis	Negative	Positive	98.5
Infection	*Ureaplasma urealyticum*	Negative	Positive	71.7
Infection	*Ureaplasma urealyticum*	Positive	Positive	54.8
	Ureaplasma parvum	Positive	Negative	
Infection	*Candida albicans*	Negative	Positive	51.1
Infection	*Fusobacterium nucleatum*	Positive	Positive	25.9
	Bacteroides ureolyticus	Positive	Negative	
Infection	*Ureaplasma urealyticum*	Positive	Positive	24.8
Infection	*Streptococcus agalactiae*	Positive	Positive	24.6
	Ureaplasma urealyticum	Positive	Positive	
	Ureaplasma parvum	Positive	Negative	
Infection	*Candida albicans*	Negative	Positive	24.0
	Unknown bacterium	Positive	Negative	
Infection	*Ureaplasma urealyticum*	Positive	Positive	22.9
	Ureaplasma sp	Positive	Negative	
Infection	*Sneathia sanguinegens*	Positive	Negative	21.3
Infection	*Bacteroides hemolyticus*	Positive	Negative	21.1
Infection	*Fusobacterium nucleatum*	Positive	Negative	20.6
	Bacteroides ureolyticus	Positive	Negative	

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(continued)

TABLE 2
Findings in cases with microbial invasion of amniotic cavity *(continued)*

Group classification	Organism identified	16S-ribosomal DNA result	Culture result	Amniotic fluid interleukin-6, ng/mL
Infection	*Listeria monocytogenes*	Positive	Negative	20.5
	Ureaplasma sp	Negative	Positive	
Infection	*Bergeyella zoohelecum*	Positive	Negative	20.4
	Bergeyella sp	Positive	Negative	
	Fusobacterium sp	Negative	Positive	
	Clostridium sp	Negative	Positive	
Infection	*Streptococcus agalactiae*	Positive	Positive	20.4
Infection	*Staphylococcus hemolyticus*	Positive	Positive	20.3
Infection	*Leptotrichia amnionii*	Positive	Negative	19.7
Infection	*Ureaplasma urealyticum*	Positive	Positive	19.5
	Ureaplasma parvum	Positive	Negative	
Infection	*Streptococcus agalactiae*	Positive	Positive	19.3
Infection	*Ureaplasma urealyticum*	Positive	Positive	12.8
	Ureaplasma parvum	Positive	Negative	
Colonization-only	*Ureaplasma urealyticum*	Negative	Positive	1.8
Colonization-only	*Ureaplasma urealyticum*	Negative	Positive	1.0
Colonization-only	*Ureaplasma urealyticum*	Negative	Positive	0.6
Colonization-only	Unknown bacterium	Positive	Negative	0.4

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shipped to the central laboratory at ProteoGenix, Inc (Costa Mesa, CA).

If delivery occurred at the study hospital, a full-thickness placental biopsy that included chorionic plate and a segment of umbilical cord within 2-3 cm from the placental insertion site were obtained and fixed in formalin. Biopsy specimens were interpreted by a placental pathologist (T.K.M.) who was blinded to clinical and laboratory findings.

Additional specimens that were obtained as part of the parent study but were not considered for the present study included samples of maternal plasma, cervicovaginal secretions, urine at enrollment, and cord blood at delivery.

Treatment

Management of preterm labor, duration of hospitalization, route of delivery, diagnosis and management of infection, and other clinical decisions were left to the discretion of the caregivers and not specified by the protocol. The only study-specific procedures were the collection of specimens: the extra aliquot of amniotic fluid, cervicovaginal swabs, maternal plasma and urine, cord blood, and placental biopsy specimens. Caregivers had access to local hospital laboratory results but were blinded to all results from the central laboratory.

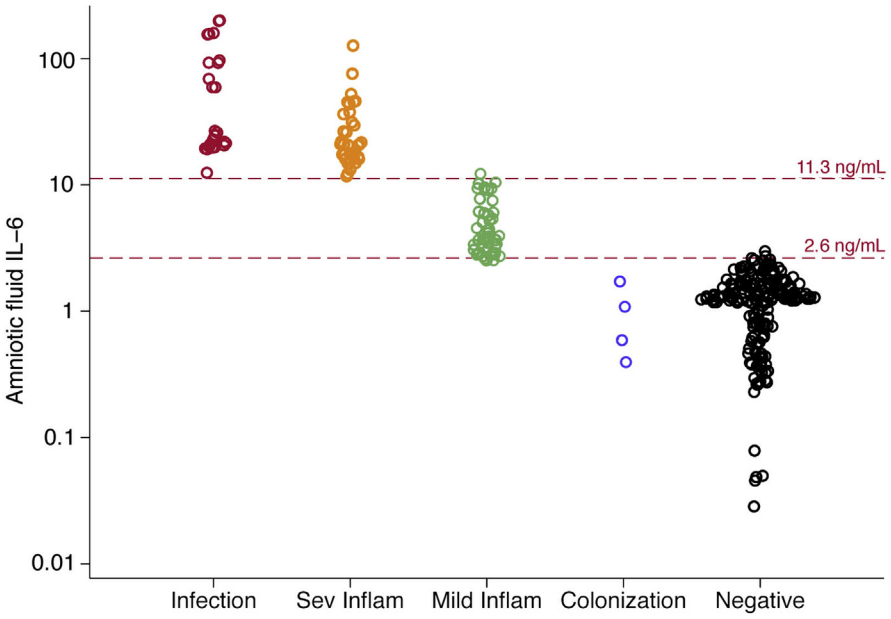
Central laboratory methods

Amniotic fluid IL-6 concentration was assayed with a quantitative sandwich enzyme immunoassay (Quantikine ELISA #D6050; R&D Systems, Minneapolis, MN).

Cultures of amniotic fluid were performed on an aliquot of amniotic fluid that had been sent to reference laboratories at the University of Washington (through May 2008) or Focus Diagnostics Inc (Cypress, CA; after June 2008). Broth enrichment techniques were used to detect low levels of aerobic and anaerobic bacteria, including genital mycoplasmas, as described elsewhere.[2]

PCR for amniotic fluid 16S rDNA was performed on DNA that was extracted with the use of an FFTE kit and Maxwell 16 system (Promega, Madison, WI). The optimized PCR reaction included universal 16S rDNA primers 5F and 531R (Invitrogen Life Technologies, Carlsbad, CA). All extraction runs included known positive and negative controls that were generated by spiking the water sample with *Staphylococcus aureus*. Positive samples, which were identified by the presence of amplicon of 460-560 base pair, were ligated into the pCR4 TOPO vector and transformed into *Escherichia coli* TOP10 cells with the use of the TOPO-TA cloning kit (Invitrogen Life Technologies). After overnight incubation on ampicillin-containing agar, up to 24 clones per sample were selected. Plasmids were isolated from bacterial colonies with the QIAprep Spin kit (Qiagen Sciences, Germantown, MD). Inserts from 24 plasmids that were generated from each positive PCR were sequenced with an AB

FIGURE 1
Dot-plot of amniotic fluid IL-6 levels in the 5 groups (log scale)



IL-6, interleukin 6; *Inflam*, inflammation; *Sev*, severe.

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3730xl sequencer (Applied Biosystems, Foster City, CA) with the custom primers Bac16S_8F2 and Bac16S_R518. For bacterial identification, bidirectional sequences were processed with MicroSeq analysis software (Applied Biosystems), and MicroSeq ID 16S rDNA 500 Library v2.0. *Ureaplasma urealyticum* and *Sneathia sanguinegens* sequences were added to create custom libraries.

Groups defined by amniotic fluid results

To define groups, we used the cut-off amniotic fluid IL-6 concentrations of 11.3 ng/mL[5,18] and 2.6 ng/mL[3] that had been determined by previous investigators. MIAC was defined by a positive 16S rDNA result and/or a positive culture from either the local hospital and/or reference laboratory.

For analysis, cases were divided into 5 groups based on amniotic fluid results: (1) infection group: MIAC plus IL-6 concentration of ≥11.3 ng/mL; (2) severe inflammation group: no MIAC, IL-6 concentration of ≥11.3 ng/mL; (3) mild inflammation group: no MIAC, IL-6 concentration of 2.6-11.2 ng/mL; (4) colonization group: MIAC, IL-6 concentration of <2.6 ng/mL; and (5) negative group: no MIAC, IL-6 concentration of <2.6 ng/mL. These groups were compared regarding baseline characteristics, pregnancy outcomes, and neonatal outcomes.

Definitions

Clinical chorioamnionitis was defined as antenatal maternal fever (≥100.4°F) plus either maternal leukocytosis (WBC >15,000/mm^3), uterine tenderness to palpation, and/or fetal tachycardia (baseline fetal heart rate >160 beats/min).

Histologic chorioamnionitis was defined by the presence of neutrophils that marginated into the placental chorionic plate; *funisitis* was defined by the presence of neutrophils in the umbilical cord vessel walls.[28]

Composite perinatal morbidity and death was defined as any ≥1 the following events: stillbirth, neonatal death, respiratory distress syndrome, grade 3 or 4 intraventricular hemorrhage, necrotizing enterocolitis, or culture-positive neonatal sepsis. Neonatal outcomes were assessed from birth to 28 days of life or until hospital discharge, whichever came first.

Statistical analyses

Between-group differences were tested with χ^2 or Fisher exact test for categoric variables, *t* test and analysis of variance for continuous variables (with log-transformation as appropriate), and log-rank test for time-to-event curves. Multivariable logistic regression analyses were performed to examine predictors of short latency, composite perinatal morbidity and mortality rates, or composite morbidity and mortality rates that excluded respiratory distress syndrome and were adjusted for gestational age at enrollment or gestational age at delivery. One set of regression models included MIAC (present or absent) and amniotic fluid IL-6 concentration (≥11.3, 2.6-11.2, or <2.6 ng/mL) as predictors. Another set of models included the 5 defined groups as predictors. Probability values of < .05 were considered significant.

RESULTS

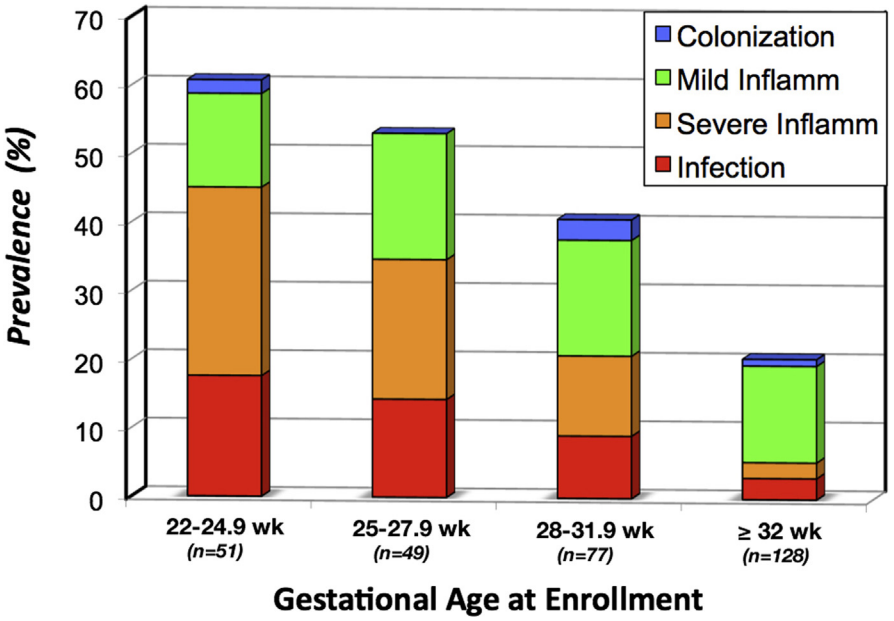
From September 2007 through November 2009, 338 women who met all the inclusion criteria were enrolled at 16 sites. Of these, 18 women withdrew or were lost to follow-up evaluation, and 15 were excluded because of inadequate specimens, which left 305 women for the analyses presented here. The number of women at each site and the gestational ages at amniocentesis are tabulated in Table 1.

Groups defined by amniotic fluid results

MIAC was found in 31 of 305 women (10.1%). As summarized in Table 2, 20 of these cases (65%) were both 16S rDNA PCR-positive and culture-positive; 6 cases (19%) were PCR-positive but culture-negative, and 5 cases (16%) were culture-positive but PCR-negative.

The distribution of amniotic fluid IL-6 concentrations that was used in the designation of the 5 groups is shown in Figure 1. IL-6 concentration of ≥11.3 ng/mL was found in 63 cases (20.7%),

FIGURE 2
Prevalence of infection, inflammation, and colonization vs gestational age



$P < .0001$ for χ^2 test across 5 groups (including the negative group, not shown).

Inflam, inflammation.

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which included 27 cases with MIAC (infection group) and 36 cases without MIAC (severe inflammation group); IL-6 concentration of 2.6-11.2 ng/mL (mild inflammation group) was found in 47 cases (15.4%), none of whom had MIAC; and IL-6 concentration of <2.6 ng/dL was found in 195 cases (63.7%), which included 4 cases with MIAC (colonization group) and 191 cases without MIAC (negative group).

Baseline characteristics

The infection and severe inflammation groups were more prevalent at earlier gestational ages, as shown in Figure 2, whereas the mild inflammation and colonization groups showed no such trend. Other than the differences in gestational age, the initial clinical presentation was similar in all 5 groups (Table 3). There was no significant difference in maternal age, rate of nulliparity, or cervical dilation. More than 80% of subjects presented with cervical dilation of ≥2 cm. Clinical signs of infection (such as fever, uterine tenderness, maternal leukocytosis, and fetal tachycardia) were uncommon in all 5 groups.

Amniotic fluid glucose level of <20 mg/dL was highly sensitive for the infection group (96%), but not for the inflammation groups (severe inflammation group, 25%; mild inflammation group, 13%), and had a false-positive rate of 29% (17 of 58 cases with glucose levels of <20 mg/dL were in the negative group). The cutoff value of 20 mg/dL was chosen because some local laboratories do not report a specific value <20 mg/dL because of known nonlinearity in the glucose assay below this level.

Gram stain was highly specific for MIAC (positive predictive value, 93%; 14 of 15), but sensitivity was only 45% (14 of 31).

Amniotic fluid WBC could not be tabulated meaningfully because of major differences in the reporting of results by local hospital laboratories: some laboratories reported total WBC; some laboratories reported only neutrophils, and some laboratories used semiquantitative scales such as 1+, 2+.

Outcomes

Survival curves that show latency are plotted in Figure 3. The infection and severe inflammation groups had similar short latencies (median, <1 and 2 days, respectively), compared with the 2 groups with low IL-6 concentrations (colonization and negative fluid groups; median, 23.5 and 25 days, respectively). The group with intermediate IL-6 concentration (mild inflammation group) had intermediate latency (median, 7 days). Cases in the infection group had the highest rates of histologic chorioamnionitis and funisitis (Table 4). Clinical chorioamnionitis and postpartum endometritis were uncommon in all 5 groups.

Perinatal outcomes are summarized in Table 5. The infection and severe inflammation groups had similar high rates of perinatal morbidity and mortality; the mild inflammation group had an intermediate rate of perinatal morbidity and mortality.

Logistic regression analyses were performed to examine whether MIAC or amniotic fluid IL-6 had associations with either latency of <72 hours or composite perinatal morbidity and death that were independent of gestational age. As shown in model 1 in Table 6, when adjusted for gestational age at enrollment, both MIAC and IL-6 levels had significant associations with latency of <72 hours, but only IL-6 levels had significant associations with composite perinatal morbidity and death. In model 2, the 5 amniotic fluid groups had strong associations with both latency and composite morbidity and death ($P <$ .0001 for the across-category trends). We also performed logistic regression analyses adjusting for gestational age at delivery rather than gestational age at enrollment. In these analyses, the gestational age variable dominated, and there was no significant additional contribution of MIAC, amniotic fluid IL-6 (model 3), or the 5 amniotic fluid groups (model 4).

Comment

Our results confirm previous observations that women in preterm labor have

TABLE 3
Clinical characteristics at enrollment

Variable	Group: Amniotic fluid interleukin-6, ≥11.3 ng/mL: Infection (n = 27)	Amniotic fluid interleukin-6, ≥11.3 ng/mL: Severe inflammation (n = 36)	Amniotic fluid interleukin-6, 2.6-11.2 ng/mL: Mild inflammation (n = 47)	Amniotic fluid interleukin-6, <2.6 ng/mL: Colonization (n = 4)	Amniotic fluid interleukin-6, <2.6 ng/mL: Negative (n = 191)	*P* value[a]
Demographics						
Maternal age, y[b]	25.8 ± 5.4	26.6 ± 4.9	26.5 ± 5.2	25.5 ±5.2	25.8 ±5.6	.92
Nulliparous, n (%)[c]	10 (37)	11 (31)	19 (41)	2 (50)	48 (25)	.17
Presentation						
Gestational age, wk[b]	27.5 ± 3.9	26.8 ± 3.5	29.9 ± 3.9	30.3 ± 4.7	31.1 ± 3.6	< .0001
Cervical dilation, cm[b,d]	3.0 ± 1.6	3.2 ± 1.4	3.3 ± 1.5	2.0 ± 1.4	2.7 ± 1.4	.05
Cervical dilation ≥2 cm, n (%)[d]	21 (88)	27 (90)	39 (85)	2 (50)	134 (80)	.27
Maternal signs, n (%)						
Uterine tenderness	2 (7)	5 (14)	2 (4)	1 (25)	10 (5)	.13
Fever ≥100.4°F	1 (4)	0	0	1 (25)	8 (4)	.10
White blood cell count >15,000/mm^3	13 (48)	14 (39)	5 (11)	2 (50)	22 (12)	< .001
Baseline fetal heart rate >160 beats/min	1 (4)	0	0	0	4 (2)	.54
Amniotic fluid markers						
Glucose <20 mg/dL, n (%)[e]	25 (96)	9 (25)	6 (13)	1 (33)	17 (9)	< .0001
Gram stain positive, n (%)	14 (52)	0	0	0	1 (<1)	< .0001
Amniotic fluid interleukin-6, ng/dL[f]	41.4 ± 2.5	23.0 ± 1.7	4.4 ± 16	0.8 ± 1.9	1.0 ± 2.4	N/A

N/A, analysis of variance was not performed because amniotic fluid interleukin-6 was used to define the groups; the difference between the infection and severe inflammation groups was significant at $P = .002$ (*t* test on log-transformed values).

[a] Comparison of subjects across all 5 groups with analysis of variance or χ^2 test; Fisher exact test was used in lieu of χ^2 for clinical signs of infection because of the small counts; [b] Data are given as mean ± SD; [c] N = 27, 35, 46, 4, and 191 for the 5 groups, respectively, because of missing values; [d] N = 24, 30, 46, 4, and 167 for the 5 groups, respectively, because of missing values; [e] N = 3 for the colonization group and n = 190 for the negative fluid group because of missing values; [f] Data are given as geometric mean and SD.

Combs. Inflammation, infection, preterm labor. Am J Obstet Gynecol 2014.

high rates of intraamniotic infection and inflammation, especially at early gestational ages.[2-4] We also confirm that intraamniotic inflammation, which is evidenced by high IL-6 levels, is associated with short latency and high rates of perinatal morbidity and mortality whether or not microbes are detected in the amniotic fluid.[3,4]

We present 4 novel findings: (1) In the inflammation groups, the absence of MIAC was shown not only by negative amniotic fluid cultures as in previous reports but also by negative 16S rDNA PCR. (2) The degree of inflammation (mild or severe) as categorized by a single biomarker (IL-6 concentration) correlated with the rate of perinatal morbidity and mortality. (3) The amniotic fluid colonization group had similar outcomes to the negative group. (4) Amniotic fluid IL-6 level was stronger than MIAC as a predictor of composite perinatal morbidity and death in the logistic regressions, but IL-6 concentration was no longer predictive after adjustment for gestational age at delivery.

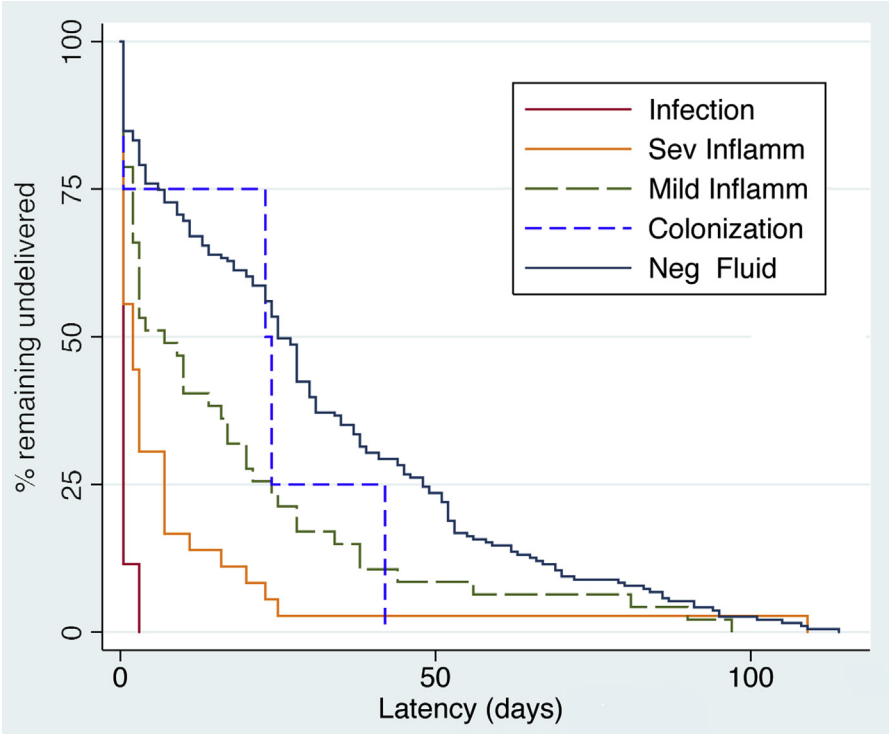
Intraamniotic infection (MIAC plus high IL-6)

As in previous reports, we found that most cases of intraamniotic infection are occult, that is, not associated with clinically evident chorioamnionitis.[2-4,19,22,23,25] In some of these cases, the short latency may have been a reflection of caregivers' responses to amniotic fluid Gram stains, glucose levels, WBC, or other signs of infection. In the remainder, however, the short latency likely reflected the natural history of intraamniotic infection.

Intraamniotic inflammation without MIAC

We confirm previous findings that culture-negative intraamniotic inflammation is associated with short latency

FIGURE 3
Kaplan-Meier time-to-event curves showing latency to delivery



Between group differences, $P < .0001$, log-rank test.

Inflamm, inflammation; *Neg*, negative; *Sev*, severe.

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and perinatal morbidity and death.[3-5,18] However, a negative culture does not exclude MIAC adequately because many intraamniotic bacteria in preterm labor are resistant to culture.[19-26] In the present study, we evaluated amniotic fluid by both sensitive culture techniques and by 16S rDNA. Previous investigators found that 16S-positive cases had similar outcomes to culture-positive cases; however, they did not report the outcomes of those with elevated inflammatory markers but who were negative for both 16S PCR and culture as a distinct group.[20,24,25] We found that this group (severe inflammation group) had similar outcomes to those with intraamniotic infection. The short latency in these cases must reflect the natural history of intraamniotic inflammation and not the caregivers' responses to the diagnosis of inflammation because caregivers had no access to IL-6 results.

What triggers intraamniotic inflammation in the absence of intraamniotic microbes? One possibility is that inflammation is the response to microbial invasion of extraamniotic compartments such as the decidua,[29] membranes,[8,11,30] or placenta.[31,32] Another possibility is that the inflammatory response might be triggered by noninfectious insults such as trauma, ischemia,[33] or abruption.[34]

We confirm an earlier report that the outcomes of intraamniotic inflammation are not "all-or-nothing" but have grades of severity that correlate with the levels of intraamniotic inflammatory mediators.[27] The previous report based severity on the number of biomarkers that were present (severe inflammation if 3 or 4 markers; minimal inflammation if 1 or 2 markers). We find that severity can be based instead on the concentration of a single marker, amniotic fluid IL-6. High levels of IL-6, with MIAC (infection group) or without MIAC (severe inflammation group), were associated with short latencies and high rates of perinatal morbidity and mortality, whereas intermediate levels of IL-6 (mild inflammation group) were associated with intermediate latency and perinatal morbidity and death. These findings are supported by another recent report that concluded that IL-6 alone or MMP-8 alone had equivalent diagnostic performance to the 4-biomarker scoring system in the identification of MIAC or intraamniotic inflammation.[35]

It is likely that mild inflammation in our cases represents an early stage in a process that progresses to severe inflammation over a period of days to weeks. In a monkey model of preterm labor that was induced by intraamniotic inoculation of bacteria, for example, levels of bacteria and cytokines rise progressively over time and correlate with the onset of uterine contractions.[36-39] However, it is also possible that mild inflammation might remain mild over time and simply have a slower time-course of progression to delivery. Our data do not allow us to distinguish these possibilities because we did not perform serial amniocenteses.

Colonization of amniotic fluid

Our finding of a few cases of amniotic fluid colonization is consistent with published observations. In Table 7, we compiled 16 studies of preterm labor that reported on both MIAC and at least 1 amniotic fluid marker of inflammation.[3-7,9,11,12,15-17,20,23,26,40] Ten of the studies documented occasional cases that we would classify as colonization (that is, MIAC with low levels of inflammatory markers). When we pooled the data, colonization was found in slightly >1% of all cases of preterm labor or nearly 9% of cases with MIAC. None of the other tabulated studies reported outcomes of the colonization cases. We found that those with colonization had outcomes similar to those with negative fluid, although our statistical power is limited by the small number of cases. *U urealyticum* accounted for 3 of our 4 cases of colonization. This organism is

TABLE 4
Pregnancy outcomes

Variable	Group: Amniotic fluid interleukin-6, ≥11.3 ng/mL: Infection (n = 27)	Amniotic fluid interleukin-6, ≥11.3 ng/mL: Severe inflammation (n = 36)	Amniotic fluid interleukin-6, 2.6-11.2 ng/mL: Mild inflammation (n = 47)	Amniotic fluid interleukin-6, <2.6 ng/mL: Colonization (n = 4)	Amniotic fluid interleukin-6, <2.6 ng/mL: Negative (n = 191)	*P* value[a]
Gestational age at delivery, wk[b]	27.6 ± 3.9	27.9 ± 3.9	32.3 ± 4.7	33.6 ± 4.4	35.5 ± 3.3	< .0001
Median latency, d[c,d]	<1 (0–1)	2 (0–7)	7 (2–24)	23.5 (11.5–33)	25 (6–48)	< .0001
Latency <72 h, n (%)[c]	23 (88)	20 (56)	16 (34)	1 (25)	32 (17)	< .0001
Latency <7 d, n (%)[c]	26 (100)	25 (69)	23 (49)	1 (25)	48 (25)	< .0001
Birth <37 wk, n (%)[c]	26 (100)	35 (97)	39 (83)	3 (75)	122 (64)	< .0001
Clinical chorioamnionitis, n (%)	4 (15)	2 (6)	2 (4)	1 (25)	11 (6)	.17
Histologic chorioamnionitis, n (%)[e]	22 (85)	13 (38)	7 (19)	0	10 (8)	< .0001
Funisitis, n (%)[e]	19 (73)	7 (20)	7 (19)	0	13 (11)	< .0001
Postpartum endometritis, n (%)	1 (4)	1 (3)	1 (2)	1 (25)	6 (3)	.26

[a] Comparison of subjects across all 5 groups with analysis of variance (gestational age), nonparametric analysis of variance (latency), or χ^2 test for other outcomes; for histologic chorioamnionitis, funisitis, and postpartum endometritis, the Fisher exact test was used because of small sample sizes; [b] Data are given as mean ± SD; [c] N = 26 in the infection group because of missing data; [d] Data are given as median (interquartile range) for latency; [e] N = 26, 34, 37, 1, and 120 for the 5 groups, respectively, because of missing data.

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common in genital tract flora and unlikely to be a skin contaminant.

Recent evidence challenges the traditional view that the normal intrauterine environment is sterile. With the use of 16S PCR techniques, bacteria have been found in fetal membranes,[30] placenta,[31,32] and amniotic fluid[41] in a substantial fraction of pregnancies at cesarean delivery without labor, although these findings are contradicted by others.[42,43] Amniotic fluid from second-trimester genetic amniocentesis in asymptomatic women is culture-positive in 0.3-6.6% of cases[44-47] and 16S PCR-positive in 10-12%.[48,49] Most of these women carry their pregnancies uneventfully and deliver at term without sequelae,[47-49] which suggests colonization rather than infection. Some of these women have elevated amniotic fluid inflammatory markers, and these women are at high risk of miscarriage or preterm birth.[50-54]

Inflammation as the main determinant of outcome

The logistic regressions (Table 6) demonstrate that IL-6 is more strongly correlated with latency of <72 hours and composite perinatal morbidity and death than is MIAC (models 1 and 2). In other words, these adverse outcomes are related more closely to intraamniotic inflammation than to the presence of microbes. The observation that the predictive value of IL-6 did not persist after adjustment for gestational age at delivery (models 3 and 4) suggests that the amniotic inflammatory response is associated with perinatal morbidity and death primarily because of its association with short latency and early preterm delivery rather than a direct contribution to fetal or neonatal inflammation. This would seem to refute the hypothesis that neonates who are born in the setting of intraamniotic inflammation necessarily fare worse than gestational age–matched neonates who are born without intraamniotic inflammation.

The interplay between intraamniotic bacteria and the inflammatory response can be summarized with a model that involves 4 stages: homeostasis, incitement, evolution, and resolution.[55] We propose that some microbes, especially *Ureaplasmas* and other genital mycoplasmas, may exist at low levels in the intrauterine milieu in many normal human pregnancies by colonizing the decidua, placenta, fetal membranes, and, occasionally, the amniotic fluid. If the organisms are kept in check by a low-level inflammatory response (homeostasis), such colonization has no adverse sequelae for mother or fetus. However, in some cases, the balance may be upset by the unchecked proliferation of organisms or by the invasion of more aggressive strains. Such imbalance could trigger a more vigorous inflammatory response (incitement). We propose that the inflammatory response, not the microbial invasion, triggers the release of prostanoids that cause contractions and cervical change, which are the clinical hallmarks of preterm labor (evolution). Once established, severe intraamniotic inflammation almost always progresses rapidly to delivery (resolution). It is unknown precisely what factors drive the transitions between these stages, so it is unknown whether clinical interventions at early stages might make a different resolution possible, that

TABLE 5
Perinatal outcomes

Variable	Group: Amniotic fluid interleukin-6, ≥11.3 ng/mL: Infection (n = 27)	Amniotic fluid interleukin-6, ≥11.3 ng/mL: Severe inflammation (n = 36)	Amniotic fluid interleukin-6, 2.6-11.2 ng/mL: Mild inflammation (n = 47)	Amniotic fluid interleukin-6, <2.6 ng/mL: Colonization (n = 4)	Amniotic fluid interleukin-6, <2.6 ng/mL: Negative (n = 191)	*P* value[a]
Birthweight, g[b]	1165 ± 614	1335 ± 839	2083 ± 858	2206 ± 1102	2725 ± 772	< .0001
Composite perinatal morbidity and death: ≥1 of the following, n (%)	22 (81)	26 (72)	25 (53)	1 (25)	41 (21)	< .0001
Perinatal death	5 (19)	7 (19)	3 (6)	0	3 (2)	< .0001
Stillbirth	0	3	1		2	
Neonatal death	5	4	2		1	
Respiratory distress syndrome, n (%)	17 (63)	20 (56)	23 (49)	1 (25)	37 (19)	< .0001
Intraventricular hemorrhage, grade 3 or 4, n (%)	2 (7)	3 (8)	2 (4)	0	4 (2)	.15
Necrotizing enterocolitis, n (%)	1 (4)	2 (6)	0	0	0	.02
Culture proven neonatal sepsis, n (%)	6 (22)	6 (17)	4 (9)	0	6 (3)	.001

[a] Comparison of the subjects in the 5 groups by analysis of variance (birthweight) or Fisher exact test (morbidities); [b] Data are given as mean ± SD.

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is, continuation of pregnancy rather than inexorable progression to preterm delivery.

Amniotic inflammatory response syndrome

In critical care, the term *systemic inflammatory response syndrome* is used to denote the complex multiorgan response to systemic inflammation.[56] The advantage of such a term over terms such as *sepsis* or *bacteremia* is that the terminology emphasizes that the inflammatory response is more directly responsible for much of the end-organ damage and other sequelae of infection than are the infecting organisms themselves. This helps to focus clinical attention on treatment of the host inflammatory response in addition to the simple eradication of the organisms.

The analogous term *fetal inflammatory response syndrome* describes the association between elevated fetal plasma IL-6 levels and a spectrum of severe neonatal morbidities and death.[57]

We propose the term *amniotic inflammatory response syndrome* to describe the relationship between elevated amniotic fluid inflammatory markers such as IL-6 and a spectrum of adverse outcomes that include early preterm birth and perinatal morbidity and death. The term *amniotic inflammatory response syndrome* focuses attention on intraamniotic inflammation rather than infection because the inflammatory response may be more directly responsible than the presence of microbes for a short latency and the resultant perinatal morbidity and death.

Culture vs PCR for detection of MIAC

We found some discordance between culture and PCR results. Among the cases with MIAC, 65% were positive by both culture and PCR; 16% were positive by culture only, and 19% were positive by PCR only. These rates of concordance/discordance are similar to those in a previous study: 40%, 24%, and 36%, respectively.[24]

Cases with positive culture and negative PCR are of special interest because it is often assumed that PCR is more sensitive than culture. However, various factors that include low bacterial loads or PCR inhibitors in body fluids actually may render it less sensitive. In our series, 1 case had a positive culture for *Candida*; the negative PCR in this case is not surprising because *Candida* is eukaryotic and would not be expected to have prokaryotic 16S rDNA. Both this case and 1 other with positive culture, despite negative PCR, had elevated IL-6 levels (51.1 and 71.7 ng/dL, respectively; Table 2) and were classified as infection; both women delivered within 72 hours. The other 3 women with positive cultures, despite negative PCR, all had *U urealyticum* (Table 2) with low colony counts (data not shown), had low IL-6 levels (< 1.8 ng/dL), and were classified as colonization; they had longer latencies. This does not imply that *Ureaplasma* is always benign. Indeed, several other cases had *U urealyticum*, either in isolation or in mixed culture, with elevations of IL-6 levels (ie, infection; Table 2), and all had short latency.

TABLE 6
Logistic regression analyses

	Outcome					
	Latency <72 h		Composite perinatal morbidity and mortality rate		Composite perinatal morbidity and mortality rate, excluding respiratory distress syndrome	
Predictor variables	Odds ratio	95% CI	Odds ratio	95% CI	Odds ratio	95% CI
Model 1[a]						
Microbial invasion of amniotic cavity amniotic fluid interleukin-6	4.3[b]	1.4–13.0	1.8	0.6–5.5	1.4	0.3–6.6
≥11.3 ng/mL	13.2[c]	5.5–31.8	4.9[c]	2.2–11.2	4.9[c]	1.9–12.8
2.6-11.3 ng/mL	3.2[c]	1.5–6.7	3.1[c]	1.5–6.4	2.5	0.8–7.6
<2.6 ng/mL	Referent	—	Referent	—	Referent	—
Model 2[a]						
Infection	70.5[c]	17.5–279	9.8[c]	3.3–29.5	7.0[c]	2.4–20.4
Severe inflammation	11.7[c]	4.7–29.2	4.5[c]	1.9–10.7	4.4[b]	1.6–11.8
Inflammation	3.1[b]	1.4–6.6	3.0[b]	1.5–6.4	2.2	0.8–6.7
Mild colonization	1.8	0.2–18.8	0.9	0.8–11.2	N/A	N/A
Negative	Referent	—	Referent	—	Referent	—
Model 3[d]						
Microbial invasion of amniotic cavity amniotic fluid interleukin-6			1.4	0.3–6.6	1.1	0.3–3.4
≥11.3 ng/mL			0.2	0.1–0.9	0.9	0.3–2.8
2.6-11.3 ng/mL			1.2	0.4–3.6	0.6	0.2–2.5
<2.6 ng/mL			Referent	—	Referent	—
Model 4[d]						
Infection			0.4	0.1–2.5	0.9	0.2–3.4
Severe inflammation			0.2	0.0–0.8	0.6	0.2–2.3
Mild inflammation			1.2	0.4–3.5	0.5	0.1–2.0
Colonization			0.2	0.0–16.1	N/A	N/A
Negative			Referent	—	Referent	—

N/A, not applicable.

[a] Adjusted for gestational age at enrollment; [b] $P \leq .005$; [c] $P \leq .001$; [d] Adjusted for gestational age at delivery.

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We conclude that culture and PCR are complementary techniques in the detection of MIAC and that neither technique alone can be relied on to exclude MIAC.

Strengths and limitations of the study

Strengths of the study include the large number of subjects, which provided statistical power to analyze outcomes based on different levels of amniotic fluid IL-6. Another strength is the use of both culture and 16S rDNA PCR in defining MIAC.

Limitations include the small number of subjects with colonization, which diminished statistical power to compare outcomes with the other groups. Another limitation was the nonuniform reporting of amniotic fluid WBC by different hospital laboratories, which made it impossible to combine and analyze the results. Another limitation was the inability to distinguish between various values of amniotic fluid glucose <20 mg/dL because some of the laboratories would not report specific values

TABLE 7
Reported incidence of MIAC and colonization of amniotic fluid in preterm labor with intact membranes

Reference	MIAC			Colonization	
	Detection method	n/N (%)	Threshold definition of amniotic fluid inflammation	n/N (% of series)	n/N (% of those with MIAC)
Romero et al, 1993[5]	Culture	11/120 (9.2)	IL-6 ≥11.3 ng/mL	0/120	0/11
Hillier et al, 1993[6]	Culture	9/50 (18.0)	IL-6 ≥1.5 ng/mL	0/50	0/9
Coultrip et al, 1994[7]	Culture	12/89 (13.5)	IL-6 ≥6.17 ng/mL	3/89 (3.4)	3/12 (25)
Rizzo et al, 1996[40]	Culture	18/92 (19.6)	IL-6 >2.6 ng/mL[a]	0/92	0/18
Hitti et al, 1997[20]	Culture + PCR	21/69 (30.4)	IL-6 >2.0 ng/mL	1/69 (1.4)	1/21 (5)
Greci et al, 1998[9]	Culture	6/53 (11.3)	IL-6 ≥7.586 ng/mL	1/53 (1.9)	1/6 (17)
Yoon et al, 2001[3]	Culture	21/209 (10.0)	IL-6 >2.6 ng/mL	2/209 (1.0)	2/21 (10)
Hitti et al, 2001[4]	Culture	45/151 (29.8)	Tumor necrosis factor α ≥30 pg/mL	5/151 (3.3)	5/45 (11)
Angus et al, 2001[15]	Culture	20/66 (30.3)	MMP-8 ≥0.1 ng/mL	1/66 (1.5)	1/20 (5)
Maymon et al, 2001[16]	Culture	34/378 (9.0)	MMP-8 detected	0/378	0/34
Yoon et al, 2003[23]	Culture + PCR	23/254 (9.1)	IL-6 >2.6 ng/mL[a]	3/254 (1.2)	3/23 (13)
Jacobsson et al, 2003[12]	Culture + PCR	9/87 (10.3)	IL-18 ≥1.0 ng/mL	2/87 (2.3)	2/9 (22)
Jacobsson et al, 2005[11]	Culture + PCR	6/21 (28.6)	IL-6 >2.6 ng/mL[a]	2/21 (9.5)	2/6 (33)
Marconi et al, 2011[26]	Culture + PCR	8/20 (40.0)	IL-6 >2.6 ng/mL[a]	0/20	0/8
Kim et al, 2012[17]	Culture	4/132 (3.0)	MMP-8 >23 ng/mL	0/132	0/4
Present study	Culture + PCR	31/305 (10.2)	IL-6 ≥2.6 ng/mL	4/305 (1.3)	4/31 (13)
Pooled data		278/2096 (13.3%)		24/2096 (1.1%)	24/278 (8.6%)

IL, interleukin; *MIAC*, microbial invasion of amniotic cavity; *MMP*, matrix metalloproteinase; *PCR*, polymerase chain reaction.

[a] Threshold not defined by original authors.

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below this level. Another limitation was that our techniques did not test for viral invasion of the amniotic cavity, which has been documented in midtrimester amniotic fluid in a small percentage of asymptomatic women.[58] However, the frequency of viral infection in preterm labor patients is unknown.

Clinical implications

The high rate of subclinical intraamniotic infection does not imply that antibiotics should be given routinely to unselected women in preterm labor. In clinical trials, empiric antibiotic therapy has been shown not to have benefit in preterm labor with intact membranes[59] and may increase the risk of neonatal necrotizing enterocolitis[60] and cerebral palsy.[61] It is unknown whether antibiotics would be beneficial if targeted to MIAC.

Research implications

Controlled clinical trials are needed to address whether targeted antibiotic therapy is beneficial for the subgroup of women in preterm labor with known MIAC. Case reports have documented that such therapy can sometimes eradicate bacteria from the amniotic fluid in patients with preterm labor,[62-64] preterm rupture of membranes,[65,66] or a sonographic short cervix,[67,68] with subsequent continuation of pregnancy for weeks or months. But it is unclear whether these cases were associated with intraamniotic inflammation (which we would classify as infection) or not (which we would classify as colonization). If the latter, our results would suggest that a benign course would have been expected even without such treatment.

Controlled clinical trials are needed to assess the possible benefits of the treatment of intraamniotic inflammation with steroids, nonsteroidal antiinflammatory drugs, and/or other immune modulators in addition to antibiotics. In animal models of infection-induced preterm birth, salutary effects have been shown with dexamethasone,[37] ibuprofen,[37] the antiinflammatory cytokine interleukin-10,[69,70] and an antagonist of Toll-like receptor 4.[71] In humans, corticosteroid treatment appears beneficial, even when given to women with clinical or histologic chorioamnionitis,[72] perhaps because the favorable antiinflammatory effects of steroids may outweigh any harm from their immunosuppressive effects.

To optimally select patients for such trials, it would first be useful to have rapid, reliable tests for intraamniotic inflammation and/or MIAC. In principle, 16S rDNA PCR could be faster than amniotic fluid culture, but this test is not readily available for clinical use. Further, our results suggest that tests to detect inflammatory mediators such as IL-6 may be more useful than tests to detect microbes. Rapid bedside tests for IL-6, MMP-8,[73] and other inflammatory mediators are theoretically possible but not yet clinically available. Tests that use proteomic profiling of amniotic fluid or cervicovaginal secretions show some promise in the prediction of MIAC or intraamniotic inflammation[74-76] but are not clinically available.

We would hypothesize that antibiotic and/or antiinflammatory treatment would be more successful if given during the stage of mild inflammation rather than severe inflammation.

Conclusion

In preterm labor with intact membranes, there is a high rate of intraamniotic infection and inflammation, especially at early gestational ages. Latency and perinatal morbidity and death are related more closely to the degree of the inflammatory response than to the presence or absence of microbes in the amniotic fluid. Microbial colonization without inflammatory response may be relatively benign. ■

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