Amniotic Fluid Interleukin-1β and Interleukin-8 Concentrations: Racial Disparity in Preterm Birth

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The purpose of this study is to examine the racial differences between interleukin (IL)–1β and IL-8 concentrations in the amniotic fluid of black and white women with spontaneous preterm birth (PTB). In this study, 350 amniotic fluid samples were collected: 165 PTB cases (<36 weeks' gestation; 52 blacks and 113 whites) and 185 controls (normal term delivery >37 weeks' gestation; 87 blacks and 98 whites). Amniotic fluid IL-1β and IL-8 concentrations were measured by immunoassay. Wilcoxon nonparametric test was performed for statistical analysis. In data stratified by race, the median IL-1β concentration was significantly higher in black cases (80 pg/mL) compared to black controls (23.7 pg/mL; $P < .0001$*), and the difference was nonsignificant in white cases (25.5 pg/mL) compared to white controls (21.3 pg/mL;* $P = .1$*). IL-8 concentration was not higher in black cases (742.2 pg/mL) compared to black controls (731.4 pg/mL;* $P = .9$*), whereas it was higher in white cases (1362.3 pg/mL) compared to white controls (533.5 pg/mL;* $P = .0005$*). Between races, IL-1β was significantly higher in blacks (*$P < .0001$*) than in whites in PTB, whereas no significant difference was noticed in IL-8 concentration between races (*$P = .1$*). In PTB, the cytokine footprint differs in the amniotic fluid between racial groups. IL-1β is higher in black and IL-8 in white PTB. These differences in the amniotic fluid cytokine concentration might not explain the racial disparity in the PTB rate, but they are suggestive of different processes of PTB in whites and blacks.*

Key words: Ethnicity, preterm labor, cytokines, prematurity, rate, inflammation.

There is a significant disparity in the spontaneous preterm birth (PTB) rate in the United States between blacks and whites.[1-5] Although both genetic and environmental factors can theoretically contribute to PTB and differences in rate among groups, the underlying pathophysiologic pathways are still unclear. One possible mechanism that may at least partially explain the differences in PTB rate is a differential immune response in blacks versus whites. This hypothesis is supported by documented differences in the fetal immune response.[6,7] Fetal membranes derived from blacks and whites respond differently to in vitro stimulation by producing different cytokine footprints. Membranes derived from whites produce more tumor necrosis factor–α, interleukin (IL)–6, and IL-10, whereas membranes derived from blacks produced more IL-1β in response to bacterial endotoxin. IL-8 concentrations do not differ between the membranes of these 2 groups.[6,7] These data suggest racial differences in the cytokine response that may help elucidate racial differences in the underlying pathophysiological pathways of PTB.

IL-1β and IL-8 may each play important roles in stimulating PTB. An increased IL-1β concentration in the amniotic fluid of women with PTB and its effectiveness in inducing prostaglandin production by intrauterine tissues, thereby initiating preterm labor, has been well documented.[8-11] In animal models, natural IL-1β receptor antagonist has been used to prevent preterm labor induced by IL-1β, supporting the role of this cytokine in PTB in laboratory animals.[12,13] IL-8 is involved in intraamniotic inflammation by facilitating polymorphonuclear leukocyte traffic to placental membranes during microbial

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invasion of the intra-amniotic cavity (MIAC).[14-16] Increased cervical and amniotic fluid IL-8 concentrations have been considered biomarkers for infection-associated PTB.[17]

The present study was undertaken to further evaluate the contribution of IL-1β and IL-8 to PTB and their potential roles in racial disparity. IL-1β and IL-8 amniotic fluid concentrations were measured in PTB, and term deliveries from blacks and whites were assessed in the case-control groups after stratification of data by race.

METHODS

The TriStar Nashville Institutional Review Board approved this study. Subjects were included in this study after providing written consent.

Subject Recruitment

All subjects were recruited at Centennial Women's Hospital in Nashville, Tennessee, between September 2003 and December 2005. Pregnant women between the ages of 18 and 40 years were enrolled after giving informed consent at the time of admission with either preterm or term labor (defined as the presence of regular uterine contractions at a minimum frequency of 2 contractions/ 10 minutes combined with documented cervical change) leading to delivery. Race was identified by self-report and confirmed by identifying the race of the mother and father of the fetus and their parents and grandparents. The use of both parental and grandparental race of the mother and father to determine grouping provides a conservative estimator of geographic origin of the study family. This is more conservative than other methods that have shown that self-description alone serves as an effective proxy of genetic structure.[18] Subjects of mixed race were excluded from the study. We define mixed race as the presence of more than 1 racial group in the parents or grandparents of the mother or father of the baby. Only blacks and whites of non-Hispanic origin were included in this study. Gestational age was determined by last menstrual period, dated and corroborated by early ultrasound.

Patients who delivered preterm (between $22^{0/7}$ weeks and $36^{0/7}$ weeks) were included as cases. We excluded cases between $36^{1/7}$ and $36^{6/7}$ weeks to minimize overlap of case-control status. Controls were women in term labor with delivery at term (>$37^{0/7}$ weeks) with no pregnancy-related complications. Subjects with oligohydramnios or polyhydramnios, preterm premature rupture of the membranes, multiple gestations, preeclampsia, placental previa, fetal anomalies, or medical/surgical complications of pregnancy or surgeries during pregnancy were excluded. Subjects who had any surgical procedures during pregnancy or who were treated for preterm labor or for suspected intra-amniotic infection and delivered at term were excluded from the control groups; but those who were treated and delivered preterm were included as cases.

Collection of Amniotic Fluid Samples

Amniotic fluid samples were collected by transvaginal amniocentesis during active labor (either preterm or at term) before rupture of the membranes. Samples were collected before preterm or term vaginal deliveries by puncture of intact membrane using a 22-gauge needle prior to artificial rupture of the membranes through the dilated cervical os. Most of our amniotic fluid specimens (approximately 70%) were collected by transvaginal amniocentesis, whereas about 30% were collected at the time of cesarean delivery prior to artificial rupture of membranes. Direct sampling through intact membrane prior to rupture avoided contamination of amniotic fluid from vaginal and cervical fluids as much as experimentally possible. Amniotic fluid was centrifuged immediately for 10 minutes at 2000*g* to remove cellular and particulate matter. Aliquots of amniotic fluid were stored at −70°C until analysis.

Data were collected on age and socioeconomic status (education, yearly income, insurance status, and marital status) along with a complete medical and obstetric history.

Histologic examination of the placenta and umbilical cord was performed on cases, and these data were obtained from patients' medical records. Histologic chorioamnionitis was diagnosed as the presence of dense polymorphonuclear leukocyte/neutrophil (PMN) infiltration of the amniochorionic membrane (>5 PMNs/field), excluding deciduas, and as funisitis when inflammation in 1 or more of the umbilical cord vessels (vasculitis) with or without inflammation in the Wharton jelly was present. Clinical chorioamnionitis was defined as any 3 of the following: increased cell count >15 000 cells/mm^3, increased C-reactive protein levels (≥0.8 U), fever ≥102.0°F, abdominal pain and/or uterine tenderness, or foul-smelling vaginal discharge as verified from case records.[19,20] MIAC was defined by the presence of bacteria in the amniotic fluid detected by amplification of microbial 16s ribosomal DNA by polymerase chain reaction (PCR; TaqMan Assay; Applied Biosystems, Foster City, CA).[21,22] PCR data were correlated with the presence of histologic and clinical evidence of chorioamnionitis. PCR was performed

Table 1. Demographic Data Comparison Between Cases and Controls

Characteristic	Whites		*P* Value, Case vs Control	Blacks		*P* Value, Case vs Control	*P* Value, Black vs White Cases	*P* Value, Black vs White Controls
	Controls	Cases		Controls	Cases			
Maternal age, y (range)	28 (18-40)	28 (18-40)	.4	25 (18-40)	25 (18-38)	.4	.08	.0001
Apgar score at 1 min (range)	9 (4-9)	8 (1-9)	<.0001	8 (2-9)	8 (1-9)	.001	.2	.02
Apgar score at 5 min (range)	9 (8-10)	8 (1-9)	<.0001	9 (7-9)	9 (6-9)	.004	.01	.8
Gestational age, d (range)	273 (253-292)	237.5 (154-253)	<.0001	273 (259-292)	237 (161-252)	<.0001	.5	.5
Birth weight, g (range)	3355 (2340-4252)	2074 (370-3790)	<.0001	3278 (2075-4382)	2177 (746-3350)	<.0001	.5	.006
Gravidity, n (%)	2 (1-8)	2 (1-6)	.01	3 (1-7)	2 (1-6)	.7	.001	.2
Latency, d (range)	*	5 (0-38)	—	*	6 (0-80)	—	0.1	—

These tables are based on available data. Latency, days between reported preterm contraction and delivery.
*Latency in controls is not shown as all of them were less than 24 hours.

in all cases, with a subgroup of controls to rule out contamination of bacterial colonizers during collection of samples. Bacterial species identification was not made.

Multiplex Assay for IL-1β and IL-8

IL-1β and IL-8 concentrations in the amniotic fluid were measured with multiple solid-phase sandwich immunoassays, using antibody-coated beads (Biosource International, Camarillo, CA) and analyzed with a Luminex instrument (Austin, TX). Five other analytes were also measured in the amniotic fluid along with IL-1β and IL-8. Standard curves were developed using duplicate samples of known quantities of recombinant proteins provided by the manufacturer. Sample concentrations were determined by relating the absorbance obtained to the standard curve by linear regression analysis. When necessary, samples were diluted in assay buffer provided by the manufacturer in addition to the doubling dilution required to perform the assay. The sensitivity of the assay was 1 pg/mL for IL-1β and 3 pg/mL for IL-8. The interassay coefficient of variations for IL-1β and IL-8 were 8.8% and 9.8%, respectively. All values are reported in units of pg/mL.

Statistical Analysis

A normality test (Shapiro-Wilk test) was performed to assess the distribution of amniotic fluid analyte concentrations. Because the concentrations were not normally distributed, the nonparametric Wilcoxon test was used to compare the difference between values using SAS (SAS 9.1; SAS Institute Inc, Cary, NC). A *P* value of <.05 was considered significant. Demographic data were compared between groups using the same measures. Multiple regression analysis was conducted to check the relationship between cytokine concentrations and social status and clinical variables as potential confounders. Power was calculated to determine a difference of 50 pg/mL of IL-1β and 200 pg/mL of IL-8, with a power of 80% at $\alpha = .05$.

RESULTS

Three hundred fifty amniotic fluid samples were analyzed for this study, consisting of 165 cases (52 blacks and 113 whites) and 185 controls (87 blacks and 98 whites). Between the cases (blacks vs whites), there were no significant differences in any demographic or clinical measures, except gravidity, which was significantly higher in blacks ($P = .001$). All clinical and demographic characteristics are described in Table 1. In cases, the incidence of histologic chorioamnionitis, funisitis, clinical signs of chorioamnionitis (such as fever and increased C-reactive protein), MIAC, and bacterial vaginosis did not differ between blacks and whites (Table 2). A subgroup of our control samples (n = 25) were subjected to PCR-based detection of microbial DNA, and none were PCR positive, indicating that contamination of vaginal bacterial flora does not occur during sample collection. This confirms the PCR results of cases, ruling out potential false

Table 2. Clinical Data on Cases: Number of Cases in Each of the Listed Clinical Variables

	Blacks		Whites		
Preterm Birth Case Variable	**n**	**%**	**n**	**%**	***P* Value Blacks vs Whites**
Histologic chorioamnionitis	17	33	44	39	.1
Funisitis	3	6	3	3	.2
Increased C-reactive protein (>0.8 U/mL)	16	32	31	27	.7
Fever (>102°F)	9	17	20	18	.8
Microbial invasion of the amniotic cavity	17	33	30	27	.4
Bacterial vaginosis*	8	15	10	8	.4

*Bacterial vaginosis was diagnosed on gram-stained smears using criteria defined by Nugent et al.[23]

Table 3. IL-1β and IL-8 Concentration (pg/mL) in Amniotic Fluid: Data Stratified by Race

		Blacks		Whites	
	Variable	**Median**	**Interquartile Range**	**Median**	**Interquartile Range**
Cases	IL- 1β	80.0	601.9	25.5	44.3
	IL-8	742.2	4353.3	1362.3	3327.9
Controls	IL- 1β	23.7	40.8	21.3	38.5
	IL-8	731.4	1327.5	533.5	852.2

IL, interleukin.

Table 4. IL-1β and IL-8 Concentration (pg/mL) in Amniotic Fluid: Data Stratified by Race and Presence and Absence of MIAC

		Blacks		Whites	
	Variable	**Median**	**Interquartile Range**	**Median**	**Interquartile Range**
No MIAC	IL- 1β	62.3	230.5	25.6	39.1
	IL-8	664.7	3215.6	1265.6	2954.3
MIAC	IL-1β	461.0	1193.8	28.2	141.8
	IL-8	742.3	4361.5	1615.1	4056.0

IL, interleukin; MIAC, microbial invasion of the intra-amniotic cavity.

positives. Overall, MIAC was detected in 33% of blacks and 27% of whites.

IL-1β was detectable in 96% (156/165) of cases and 97% (180/185) of control samples. IL-8 was detectable in 97% (160/165) of cases and 95% (174/185) of control amniotic fluid samples. Both amniotic fluid IL-1β and IL-8 concentrations, at the time of active labor, were elevated in pooled cases (blacks and whites) compared to pooled controls. The median IL-1β concentration was 20.0 pg/mL in cases compared to 22.2 pg/mL in controls ($P < .0001$), and the median IL-8 concentration was 1061.7 in cases compared to 615.3 pg/mL in controls ($P = .0006$).

Data stratified by race documented significantly higher concentrations of IL-1β in black cases compared to black controls ($P < .0001$; Tables 3-5), whereas no difference was seen in white cases compared to white controls ($P = .2$; Tables 3-5). The median IL-1β concentration was 3-fold higher in black cases compared to white cases ($P < .0001$). Data stratified by both race and MIAC demonstrated a nonsignificant increase in amniotic fluid IL-1β concentration in both black ($P = .3$) and white ($P = .5$) cases. Between races, no difference was observed in control samples ($P = .9$).

IL-8 concentration was not elevated in black cases compared to black controls ($P = .9$); however, it was significantly higher in white cases compared to white controls ($P < .0001$; Tables 3 and 5). The median IL-8 concentration was ~2-fold higher in white cases compared to black

Table 5. Comparison of Amniotic Fluid IL-1β and IL-8 Concentration in Black and White Cases and Controls *P* Values

	Comparisons Between Races				Comparisons Within Races			
					Blacks		Whites	
Analyte	Cases, Blacks vs Whites	Cases With MIAC, Blacks vs Whites	Cases With No MIAC, Blacks vs Whites	Controls, Blacks vs Whites	Case vs Control	Cases, MIAC vs No MIAC	Case vs Control	Cases, MIAC vs No MIAC
IL-1β, *P* value	<.0001	.01	<.0001	.9	<.0001	.3	.2	.5
IL-8, *P* value	.1	.2	.4	.2	.9	.9	<.0001	.4

IL, interleukin; MIAC, microbial invasion of the intra-amniotic cavity.

cases, although the difference between the races was not statistically significant ($P = .1$). There are no differences within cases as a function of MIAC (Tables 4 and 5). Control amniotic fluid IL-8 concentration was also not different between races ($P = .2$).

Because in extreme prematurity (<28 weeks) infection is a predominant etiological factor,[24] we analyzed the data from cases after stratification for gestational age and MIAC. Nine (17%) blacks and 15 (14%) whites were delivered at less than 28 weeks gestation. In blacks, the median IL-1β concentration for pregnancies <28 weeks was greater as compared to those >28 weeks (651 pg/mL and 49.2 pg/mL, respectively, $P = .05$). Comparable results were found for IL-8 (4178 pg/mL vs 407.2 pg/mL for <28- and >28-week pregnancies; $P = .04$). In whites, the IL-1β concentration was not different between the 2 groups (23.2 pg/mL and 25.6 pg/mL, respectively; $P = .7$); however, IL-8 was significantly higher (3960 pg/mL) in the <28-week group compared to the >28-week group (1281 pg/mL; $P = .01$).

MIAC was present in 77% of black and 60% of white pregnancies ending prior to 28 weeks but only in 23% of black and 22% of white pregnancies longer than 28 weeks. Blacks had higher IL-1β and IL-8 concentrations in the MIAC group, whereas whites showed a higher IL-8 concentration but not IL-1β concentration in the MIAC group compared to the non-MIAC group of less than 28 weeks. As hypothesized,[24] MIAC had no pronounced effect on either cytokine the >28-week groups in either race.

We also tested cytokine concentrations based on route of delivery and found no significant differences in IL-1β or IL-8 concentrations in either cases or controls (data not shown). All our cytokine data were tested for potential confounding variables (both clinical and demographic), and these were found to have no effect in either race.

DISCUSSION

Race was not identified as a risk modifier in previous reports.[10,14-16] Our exploratory analysis of pooled samples from blacks and whites was consistent with prior reports in that we saw significantly higher cytokine concentrations in cases compared to controls for both IL-1β and IL-8. However, when we analyzed samples from the 2 groups separately, we found that black cases had significantly higher concentrations of IL-1β than both white cases and black controls. White cases had higher IL-8 concentrations than white controls but did not differ significantly from black cases. MIAC was associated with increased IL-1β and IL-8 concentrations in blacks in deliveries <28 weeks; however, only IL-8 was higher in whites with MIAC in deliveries <28 weeks. Overall (in all cases), the effect of MIAC was not significant with either cytokines in both races.

These results are important for several reasons as they indicate racial differences in the inflammatory response pathway that can lead to preterm delivery. This is a relatively novel concept that can have many implications for our understanding of this disease process. This conclusion is supported by the comparison of the pooled data and the stratified data. From these 2 analyses, it is clear that IL-1β is enhanced in black cases, whereas IL-8 concentrations are higher in white cases than in white controls.

It has been shown that important regional differences exist in the chemical content of amniotic fluid obtained from the forebag as compared to the upper uterine compartment (hindbag).[25-27] It is true that most (~70%) of our control samples are collected from the forebag if the theory of hindbag and forebag compartmentalization is true at term. This adds a lot more strength and significance to our data in that the maximum concentration of inflammatory markers should be found in the forebag compartment. As we made the comparisons using controls in which maximum cytokine concentrations would be expected, we would anticipate that this should minimize the differences noted between cases and controls. Because of this, compartmentalization is not a confounder in this study; if true, it should actually reinforce our findings.

Although our overall data do not agree with prior reports indicating that MIAC increases cytokine concentrations,[12,14,15] it is possible that both reports are accurate. The difference may be attributed to the timing of sample collection. Prior studies were done on amniotic fluid samples collected by amniocentesis for diagnosis of MIAC at early stages of labor, whereas our samples are collected prior to delivery at the time of active labor. Latency in cases is 6 days in blacks and 5 days in whites, providing ample time for immune balance to occur. It very well may be true that the initial surge of cytokines will take place during the establishment of infection (as documented by prior studies), and it will be compensated by immune regulation later on (as might be in our studies). However, increased cytokine concentrations in the MIAC groups (except IL-1β in whites) <28 weeks supports the role of overwhelming inflammation in this group with early labor. Racial disparity in fetal membrane IL-1β and IL-8 response to bacterial endotoxin stimulation in vitro also supports our current findings in amniotic fluid, in which the IL-1β response was significantly higher in black membranes and IL-8 was higher in white-derived membranes.[7]

Although IL-8, a neutrophil chemoattractant and activator, was higher in white cases as compared to black cases, the differences were not quite significant between the races. In addition, no significant difference in the rate of histologic chorioamnionitis was seen between racial groups. This may be because cases with preterm premature rupture of the membranes have been excluded from our study to provide homogeneity to our case group. This condition is more likely to be accompanied by the presence of histologic chorioamnionitis and has a higher incidence in blacks.[5]

To date, there have been few studies that have addressed the hypothesis of racial differences in immune response, but where examined, it does exist.[28] The racial differences in the pathophysiological pathways may provide an understanding as to why various therapeutic interventions succeed in some people and fail in others. They may also explain differences in sensitivity to various stimuli that trigger preterm labor. If these results are true, then the implication is that there are innate and differentially distributed risk factors among populations with respect to immune responses.

Our study supports the idea that there are racial differences in physiologic responses that affect the susceptibility to PTB. These differences may be genetically programmed or may depend on environment, gene-environment, or gene-gene interactions.[29-32] It will be important to examine the ultimate causes of the differences that we report to be able to understand underlying risks and to tailor treatments. These results should provide fertile grounds for further research in this area.

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