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Placental Features of Chorioamnionitis Colonized With *Ureaplasma* Species in Preterm Delivery

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ABSTRACT: *Ureaplasma* spp. is detected in the urogenital tract, including the vagina, cervix, chorioamnion, and placenta. Their colonization is associated with histologic chorioamnionitis (CAM), often observed in placentas from preterm delivery. We isolated *Ureaplasma* spp. from 63 preterm placentas among 151 specimens, which were delivered at <32 wk of gestation. Of the 63 placentas, 52 (83%) revealed CAM in cultures positive for *Ureaplasma* spp., however, CAM was observed only in 30% (26/88) of cultures negative for *Ureaplasma* spp. ($p < 0.01$). Colonization by *Ureaplasma* spp. was an independent risk factor for CAM (OR, 11.27; 95% CI, 5.09–24.98). Characteristic neutrophil infiltration was observed in the amnion and subchorion (bistratified pattern) in cultures positive for *Ureaplasma* spp. FISH analysis of CAM placenta with male infant pregnancy indicated that bistratified infiltrated neutrophils showed the XX karyotype and umbilical vein infiltrated neutrophils showed XY karyotype. The distribution of sulfoglycolipid, the receptor of *Ureaplasma* spp., was mainly detected in the amnion. Ureaplasmal urease D protein and *ureB* gene were both detected in the amnion, indicating direct colonization by *Ureaplasma* spp. **(*Pediatr Res* 67: 166–172, 2010)**

Ureaplasma spp. is the smallest self-replicating organism, both in genome size and in cellular dimensions. It lacks cell walls and exists in association with eukaryotic cells, mainly colonizing mucosal surfaces of the respiratory and urogenital tracts (1). *Ureaplasma* spp. is a common inhabitant of the lower genital tract and isolated from 40 to 80% women of child-bearing age (2). However, once *Ureaplasma* spp. spreads from the lower genital tract into the body, this microorganism exerts widespread pathogenic effects, such as chorioamnionitis (CAM), urinary tract infections, preterm labor, and spontaneous abortion. On the other hand, *Ureaplasma* spp. infection is also reported as a risk factor for lethal pneumonia, chronic lung disease, and meningitis of fetuses and neonates (3).

CAM is a placental finding associated with premature rupture of membranes (PROM) and preterm birth, which are the most important causes of perinatal morbidity and mortality (4,5). Previous studies showed that CAM was positively related to the isolation of *Ureaplasma* spp (6,7). Although many researchers reported the detection of *Ureaplasma* spp. from specimens of vagina, cervix, chorioamnion, and placenta using culture or PCR methods (8–12), the precise pathologic findings of CAM with *Ureaplasma* spp. remain unclear.

A variety of infectious microorganisms use specific host cell surface molecules as receptors. Such receptors provide a mechanism for intimate interaction with the host cell membrane and in some cases may facilitate the subsequent entry of the organism into the cell (13). *Ureaplasma* spp. and *Mycoplasma hominis* were shown to specifically recognize host cell surface glycolipids (sulfogalactoglycerolipid and the sphingolipid counterpart, sulfogalactosyl ceramide), which have been implicated in sperm-egg interactions (14). This glycolipid receptor binding may relate to the reproductive pathogenesis of these organisms. Furthermore, there are no previous reports about the specific receptor of *Ureaplasma* spp. and its distribution in the placenta.

This study was conducted to confirm the prevalence of placental *Ureaplasma* spp. in preterm delivery, and whether there is an association between *Ureaplasma* spp. and CAM. Moreover, we identified placental features that might be characteristic of ureaplasmal infection.

METHODS

Subjects and placental examination. All clinical specimens were obtained after informed consent approved by the Ethics Committee of Osaka Medical Center and Research Institute for Maternal and Child Health. In this study from January to December 2007, pathologic examinations of 151 placentas, including 67 cesarean deliveries, delivered at <32 wk of gestation were performed. As a control, 41 term placentas [mean gestational age, 39.6 wk (SD 1.1); mean birth weight, 3009 g (SD 425)], including 11 cesarean deliveries (27%), four PROM (10%), and two intrauterine growth retardation (5%), were also examined. The pathologists who examined the placentas were blinded to the results of *Ureaplasma* spp. culture. Placentas were examined according to the method of Fox and Sebire (15). The histologic criterion used for CAM was the presence of accumulated leukocytes extending through the fetal membranes using Blanc's classification (16). Umbilical vasculitis was defined as migration of fetal inflammatory cells into or through the media of the umbilical arteries or veins. The criterion used for subacute necrotizing funisitis was a typical deposition of calcification around the vessels in the umbilical cord.

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F.N. and T.H. contributed equally to this study.

Abbreviations: CAM, chorioamnionitis; **GBS,** group B *Streptococcus*; **PROM,** premature rupture of membranes; **TLR,** toll-like receptor

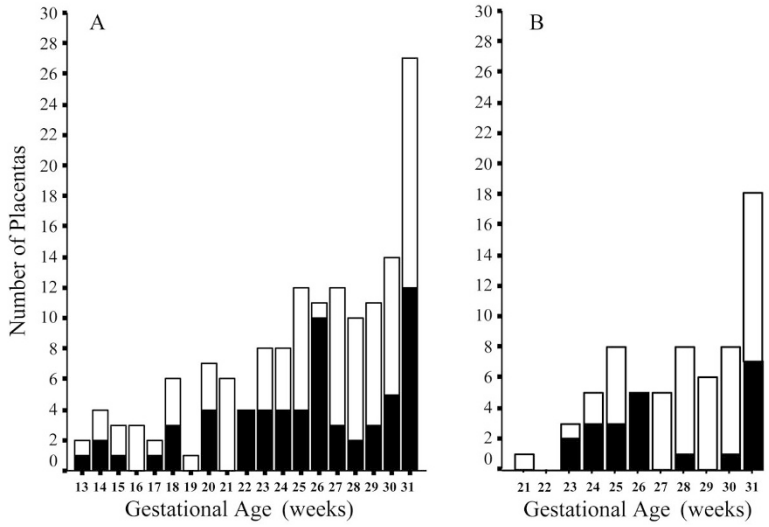

Figure 1. Isolation of *Ureaplasma* spp. from preterm placenta. Forty-two percent (63/151) of preterm placentas (<32 wk) were culture positive for *Ureaplasma* spp. (*A*), and 33% (22/67) in preterm cesarean delivery, (*B*) black bars, culture positive for *Ureaplasma* spp.; white bars, culture negative for *Ureaplasma* spp.

Culture for the detection of* Ureaplasma *spp. A microbiologist who was blinded to all clinical details of the patients prepared cultures for the detection of *Ureaplasma* spp.; species identification and subtyping of *Ureaplasma* spp. were also developed. The placental swabs were collected from the fetal side of the placentas. These were suspended in UMCHs medium: *Mycoplasma* broth base (Becton, Dickinson and Co., Baltimore, MD) 1.47% (wt/vol), yeast extract (Becton, Dickinson and Co.) 2.5% (wt/vol), horse serum (Biowhittaker, Walkersville, MD) 20% (vol/vol), supplement VX (Becton, Dickinson and Co.) 1.0% (vol/vol), urea 0.04% (wt/vol), phenol red 0.001% (wt/vol), L-cysteine hydrochloride 0.01% (wt/vol), and penicillin G 1000 U/mL. After incubation at 35°C for up to 72 h, the color of the medium changed from yellow to red due to hydrolysis of urea, and these color changes were regarded as indicating positivity for *Ureaplasma* spp. We identified *Ureaplasma* spp. by colony formation and subsequent PCR-based assays using modified Kong's method (17).

Immunohistochemistry. Paraffin-embedded sections were deparaffinized and rehydrated. The sections were incubated with rabbit anti-human myeloperoxidase polyclonal antibody (1:300; Dako, Tokyo, Japan), mouse anti-human cluster of differentiation (CD) 45 MAb (1:1; Thermo/Shandon Immunon, Pittsburgh, PA), mouse anti-human CD68 MAb (1:50; Dako), chicken anti-*Ureaplasma urealyticum* UreD polyclonal antibody (1:100; Abcam, Cambridge, MA), and mouse anti-sulfoglycolipid MAb (1:5) (18). Immunoreactivity was detected using the Envision Kit (Dako) with horseradish peroxidase-conjugated anti-chicken IgG antibody (1:200; Bethyl Laboratories, Montgomery, TX). Sections were counterstained with Mayer's hematoxylin (Muto Pure Chemicals, Tokyo, Japan).

FISH analysis. X/Y FISH was performed using the CEP X/Y DNA Probe Kit (Vysis, Downers Grove, IL) according to the manufacturer's instructions. Included in this kit are probes for Xp11.1-q11.1 of chromosome X (labeled with Spectrum Red) and for Yq12 of chromosome Y (labeled with Spectrum Green). Fluorescence microscope (Olympus IX71 microscope; Olympus, Tokyo, Japan) integrated with a digitized CCD camera (Nikon Digital Sight DS-5Mc, Nikon Instech, Kanagawa, Japan) and imaging software (Nikon ACT-2U Nikon) were used to investigate and analyze the FISH results with DAPI II counterstain.

PCR for the detection of* Ureaplasma *urease structural gene. DNA was extracted from paraffin-embedded placental sections using DNA Isolator PS-Rapid Reagent (Wako Pure Chemical Industries, Osaka, Japan) according to the manufacturer's instructions. The DNA solution was used for subsequent PCRs. The PCR primers used were as follows: *Glut1*, sense (5′-TGCAAGGGGAAAGGAAAAGG-3′) and antisense (5′-GAAGAGAAACTCTGCCCTGC-3′) of the human facilitative glucose transporter genes; and *ureB*, sense (5′-CCAGGTAA ATTAGTACCAGG-3′) and antisense (5′-CCTGATGGAATATCGAAACG-3′) of the *Ureaplasma* urease structural genes. Twenty-five micro liters of *GoTaq* Green Master Mix (Promega, Madison, WI), 1 μL sample, and 14 μL water were added to each reaction. A thermal cycler was used to process samples through 35 cycles at 95°C for 30 s, 64°C for 30 s, and 68°C for 30 s for *Glut1* and through 35 cycles at 95°C for 30 s, 45°C for 30 s, and 68°C for 30 s for *ureB*.

Statistical analysis. All statistical analyses were performed using SPSS 11.0 for Windows (SPSS, Chicago, IL). The χ^2 test or Fisher's exact test was used to compare the incidence of each placental feature between cases culture positive and negative for *Ureaplasma* spp. Logistic regression models were used to examine the association between culture positivity for *Ureaplasma* spp. and the severity of CAM. The Mann-Whitney *U* test or unpaired *t* test was used to compare continuous variables. A difference was considered significant when the *p* value was <0.05. Logistic regression analysis, adjusting for the potential confounding clinical factors associated with CAM, were conducted to evaluate the independent association of *Ureaplasma* spp. colonization with CAM. The strength of association in these models is reported as the adjusted OR with the 95% CI.

Table 1. *Maternal characteristics associated with* Ureaplasma *spp. colonization*

	Ureaplasma spp. (+) (n = 63)	*Ureaplasma* spp. (−) (n = 88)	p
Gestational age (wk), median (range)	26.4 (13.0–31.9)	27.5 (13.0–31.9)	NS
Birth weight (g), median (range)	742 (0–1878)	753 (0–2280)	NS
Multiple pregnancy	9 (14%)	24 (27%)	NS
Placenta previa	0 (0%)	2 (2%)	
Abruptio placentae	6 (10%)	5 (6%)	NS
Preeclampsia	3 (5%)	15 (17%)	<0.05
Oligohydramnios	19 (30%)	23 (26%)	NS
Polyhydramnios	1 (2%)	5 (6%)	NS
PROM	28 (44%)	14 (16%)	<0.01
Cesarean section	22 (35%)	45 (51%)	<0.05
Culture positive for other microorganism	8 (13%)	5 (6%)	NS

RESULTS

Incidence of* Ureaplasma *spp. colonization in preterm placentas. Among 151 placentas delivered at <32 wk of gestation, 42% (63/151) were culture positive for *Ureaplasma* spp. Figure 1*A* shows the incidence of *Ureaplasma* spp. colonization in preterm placentas according to gestational age. Forty-nine percent (38/77) of placentas delivered during the second trimester (at 13–26 wk) and 34% (25/74) delivered during the early third trimester (at 27–31 wk) were colonized with *Ureaplasma* spp. The incidence of *Ureaplasma* spp. colonization during the second trimester was higher than that during the early third trimester, but the difference was not statistically significant (49% *versus* 34%; $p = 0.05$). The incidence of *Ureaplasma* spp. colonization in term placentas was 24% (10/41); this was significantly lower than that in

Table 2. *Other microorganisms detected in placental cultures*

Culture for *Ureaplasma* spp.	Placenta no.	Other microorganism
Positive ($n = 8$)	1	*Escherichia coli*
	2	*Pseudomonas fluorescens*
		Enterobacter cloacae
	3	*Candida glabrata*
	4	*Streptococcus mitis*
		Escherichia coli
	5	*Clostridium* spp.
	6	*Gardnerella vaginalis*
	7	*Pseudomonas fluorescens*
	8	*Chrvseobacterium indologenes*
Negative ($n = 5$)	9	Group B *Streptococcus*
	10	Group B *Streptococcus*
	11	*Escherichia coli*
		Prevotella bivia
	12	Group B *Streptococcus*
	13	Group B *Streptococcus*

Table 3. *Pathological features of placentas with and without* Ureaplasma *colonization*

	Ureaplasma spp. (+) ($n = 63$) (%)	*Ureaplasma* spp. (−) ($n = 88$) (%)	*p*
Gross findings			
Retroplacental hematoma	7 (11)	11 (13)	NS
Thrombosis	4 (6)	4 (5)	NS
Infarction	3 (5)	7 (8)	NS
Fibrin deposition	3 (5)	15 (17)	NS
Histopathological findings			
CAM	52 (83)	26 (30)	<0.01
CAM with umbilical cord inflammation	29 (46)	6 (07)	<0.01
Meconium staining	0 (0)	1 (1)	
Hemosiderin pigmentation	5 (8)	4 (5)	NS
Subacute necrotizing funisitis	4 (6)	0 (0)	
Villitis	0 (0)	3 (3)	
Fibrin deposition	0 (0)	1 (1)	
Syncytial knot	2 (3)	6 (7)	NS

preterm placentas (24% *versus* 42%; $p < 0.05$). On the contrary, 33% (22/67) of preterm cesarean delivery placentas (at 21–31 wk) were culture positive for *Ureaplasma* spp. (Fig. 1*B*).

Maternal characteristics associated with* Ureaplasma *spp. colonization. Maternal characteristics of the *Ureaplasma*-positive and -negative groups are shown in Table 1. The incidence of PROM in the *Ureaplasma*-positive group was significantly higher than that in the negative group (44% *versus* 16%; $p < 0.01$). The incidences of pre-eclampsia and cesarean section in the *Ureaplasma*-positive group were lower than those in the negative group (5% *versus* 17%, $p < 0.05$; 35% *versus* 51%, $p < 0.05$, respectively). There were no significant differences between these two groups in other maternal characteristics. Other microorganisms cultured from placentas are listed in Table 2. Among 151 placentas, only 13 (9%) were culture positive for other microorganisms, the most frequent of which were group B *Streptococcus* (GBS) and *Escherichia coli*.

Pathologic features of placentas in* Ureaplasma*-positive group. Placental findings in the *Ureaplasma*-positive and -negative groups are presented in Table 3. Placentas of the *Ureaplasma*-positive group showed a significantly higher frequency of CAM compared with the negative group (83% *versus* 30%; $p < 0.01$). CAM with umbilical cord inflammation was also more frequent in the *Ureaplasma*-positive group than in the negative group (46% *versus* 7%; $p < 0.01$). No differences were found in the percentages of any other findings between these two groups.

Table 4. *Risk factors for CAM*

	Univariate		Multivariate	
	OR	95% CI	OR	95% CI
Culture positive for *Ureaplasma* spp.	11.27	5.09–24.98	8.60	3.72–19.89
Multiple pregnancy	0.45	0.20–01.00	0.63	0.24–01.62
PROM	7.67	3.13–18.84	5.36	1.98–14.52
Culture positive for other microorganism	3.43	0.91–13.01	2.90	0.58–14.52

Table 5. *Pathological features of CAM placentas with and without* Ureaplasma *colonization*

	Ureaplasma spp. (+) ($n = 52$) (%)	*Ureaplasma* spp. (−) ($n = 26$) (%)	*p*
Severity			
Blanc I	3 (6)	9 (35)	—
Blanc II	14 (27)	8 (31)	<0.05
Blanc III	35 (67)	9 (35)	<0.01
Distribution			
Amniotic bistratified pattern	18 (35)	2 (8)	<0.05
Umbilical cord inflammation			
Umbilical vasculitis	29 (56)	6 (23)	<0.05
Subacute necrotizing funisitis	4 (8)	0 (0)	

Association of* Ureaplasma *spp. colonization with CAM. Table 4 displays ORs for the strength of association between histologic CAM and risk factors for the development of CAM. Culture positivity for *Ureaplasma* spp. and PROM were significantly associated with CAM (OR, 11.27; 95% CI, 5.09–24.98; and OR, 7.67; 95% CI, 3.13–18.84, respectively). The associations between culture positivity for *Ureaplasma* spp. and CAM and between PROM and CAM remained significant after adjustment for confounding factors in logistic regression analyses (OR, 8.60; 95% CI, 3.72–19.89; and OR, 5.36; 95% CI, 1.98–14.52, respectively).

Characteristics of CAM placentas colonized with* Ureaplasma *spp. To explore the characteristics of preterm CAM placentas colonized with *Ureaplasma* spp., we compared 52 CAM placentas in the *Ureaplasma*-positive group with 26 CAM placentas in the negative group (Table 5). Logistic regression models using Blanc I CAM as the reference showed that culture positivity for *Ureaplasma* spp. was associated with severe CAM (Blanc II and III).

Moreover, we found a characteristic pathologic finding named the "amniotic bistratified pattern," in which a stratified leukocyte infiltration in the amnion and subchorion was accompanied by necrosis of the amniotic epithelium (Fig. 2*A*), in contrast to GBS positive (control) placenta (Fig. 2*B*). This pattern was significantly more frequent in the *Ureaplasma*-positive group than in the negative group (35% *versus* 8%; $p < 0.05$). It counted 51% (18/35) of Blanc III CAM in the *Ureaplasma*-positive group. In all 20 placentas that showed

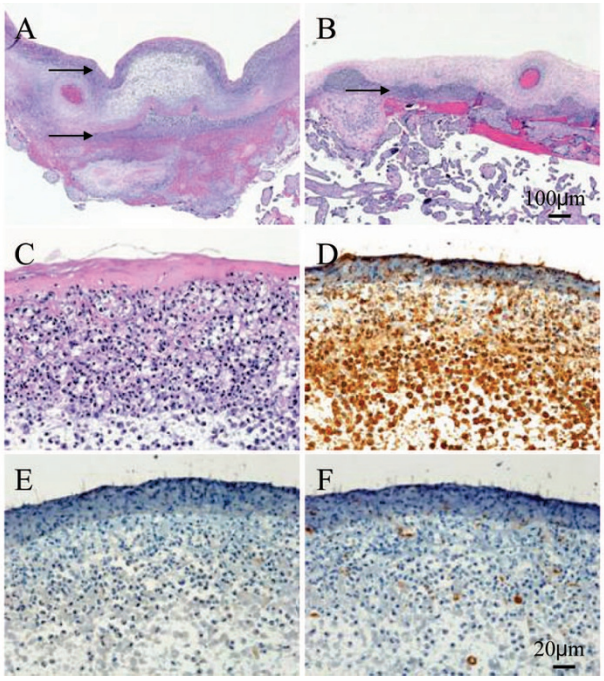

Figure 2. Amniotic bistratified neutrophil infiltration in placenta with CAM. *A*, *C–F*, Amniotic bistratified pattern in Blanc III CAM placenta colonized with *Ureaplasma* spp. *B*, Blanc III CAM placenta colonized with GBS. *A–C*, Mayer's Hematoxylin and eosin (H&E) stain. *D*, Myeloperoxidase. *E*, CD45. *F*, CD68. Arrows indicate infiltrated leukocytes.

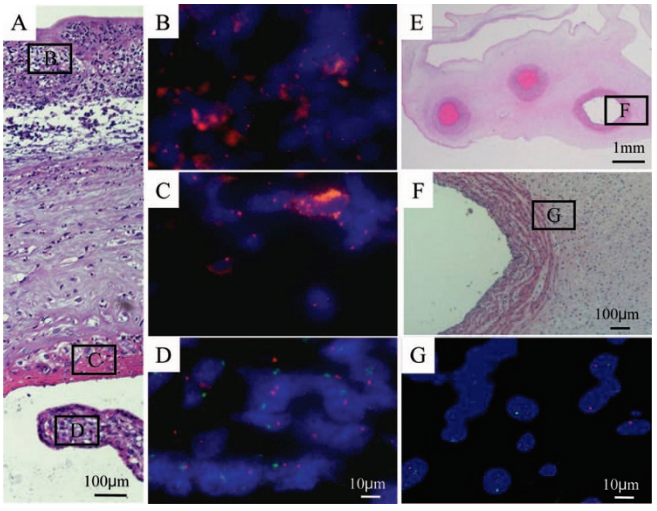

Figure 3. X(red)/Y(green) FISH analysis. (*A*) H&E stain around the membrane. X/Y FISH of (*B*) amnion, (*C*) subchorionic space, and (*D*) villi. (*E*) H&E stain of umbilical cord. (*F*) Higher magnification of umbilical vein and infiltrated cells. (*G*) X/Y FISH of infiltrated cells. One green and one red signal indicate XY karyotype (male, fetus-derived cells) and two red signals indicate XX karyotype (female, maternal-derived cells). DAPI II was used as a counterstain.

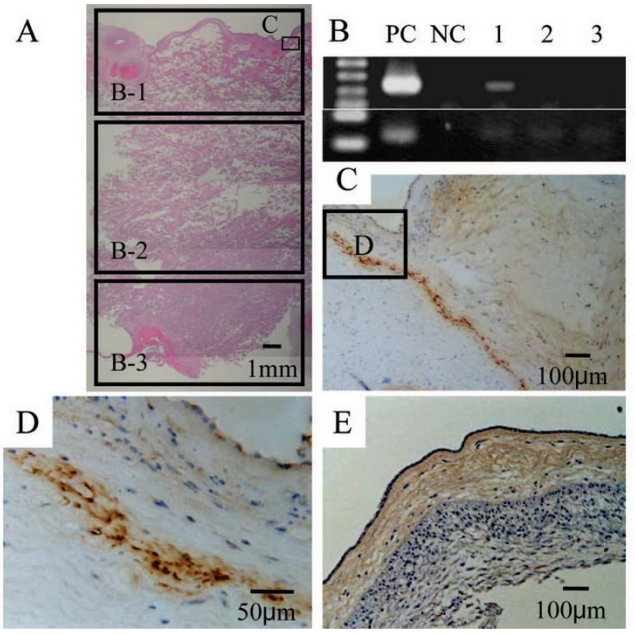

Figure 4. Distribution of *Ureaplasma* spp. and its receptor sulfoglycolipid in placenta. *A*, H&E stain. *B*, PCR for the detection of *Ureaplasma* DNA. (*Upper column*) The *Ureaplasma ureB* gene is detected in genomic DNA from chorioamnion (*lane 1*) but not in villi and decidua (*lanes 2* and *3*, respectively). PC, positive control (full *Ureaplasma* spp. genome). NC, negative control (distilled water). (*Lower column*) The human *Glut1* gene is detected in genomic DNA from chorioamnion, villi, and decidua (*lanes 1*, *2* and *3*, respectively). PC, positive control (full human genome). NC, negative control (distilled water). *C*, *D*, *Ureaplasma* UreD is detected in the amnion where maternal neutrophils had infiltrated. *E*, Sulfoglycolipid is mainly distributed in the amnion in normal term placenta.

the specific neutrophils infiltration of amniotic bistratified pattern, 13 placentas were colonized with *Ureaplasma* spp. solely, five placentas with both *Ureaplasma* spp. and other microorganisms (*Escherichia coli*, *Pseudomonas fluorescens*, *Enterobacter cloacae*, *Candida glabrata*, *Streptococcus mitis*, or *Clostridium* spp.), and two placentas without any microorganisms (Table 2). None of the placentas, which were negative for *Ureaplasma* spp. and positive for other microorganisms showed this amniotic bistratified placental pattern.

Specific placental features of intrauterine* Ureaplasma *infection. To determine the characteristics of the bistratified infiltrated cells in the amnion and subchorionic space, we analyzed *Ureaplasma*-positive Blanc III CAM placentas and GBS-positive (control) placentas by immunohistochemical staining. Most of the bistratified infiltrated cells in *Ureaplasma*-positive placentas were myeloperoxidase-positive neutrophils, indicating acute inflammation, whereas immunohistochemical staining of CD45 and CD68 were negative (Fig. 2*C–F*). Control CAM placentas with GBS infection also showed acute inflammation (data not shown).

Next, we analyzed the origin of the migrated neutrophils showing bistratified patterning around the *Ureaplasma*-positive placental membrane (Fig. 3*A*). Unexpectedly, infiltrated inflammatory cells in the amnion and the subchorionic space showed only two red signals (XX genotype), indicating maternally derived cells (Fig. 3*B* and *C*). On the other hand, trophoblasts in the villi and inflammatory cells migrating through the wall of the umbilical vessels (Fig. 3*E* and *F*) showed one red and one green signals (fetal XY genotype), as expected (Fig. 3*D* and *G*).

To explain these maternal immunologic responses, we further analyzed the localization of the microorganism's DNA in the *Ureaplasma*-positive placenta. PCR amplification of the

Ureaplasma-specific *ureB* gene was detected only in purified genomic DNA from the chorioamnion (Fig. 4*A*, *B-1*) and not detected in villi (Fig. 4*A*, *B-2*) or decidua (Fig. 4*A*, *B-3*) in the paraffin-embedded sections that showed bistratified infiltration of neutrophils (Fig. 4*B*, *upper column*). On the contrary, PCR amplification of human *Glut1* gene was detected in genomic DNA obtained from chorioamnion, villi, and decidua (Fig. 4*B*, *lower column*). By immunohistochemical staining with anti-*Ureaplasma* UreD antibody, which was a polyclonal antibody against the *Ureaplasma* urease, positive signals were detected in the amnion where maternal neutrophils had infiltrated (Fig. 4*C* and *D*). Immunohistochemical staining of sulfoglycolipid, the receptor of *Ureaplasma* spp., showed that it was mainly distributed in the amnion in normal term placenta (Fig. 4*E*).

DISCUSSION

Infectious and inflammatory processes in the uterus during pregnancy remain a major cause of preterm delivery and subsequent complications in newborn infants. Pathologic CAM is frequently associated with preterm delivery. The most common microbes isolated from the amniotic cavity of women with preterm labor are *Ureaplasma* spp. and *Mycoplasma hominis* (19). Studies based on the isolation of *Ureaplasma* spp. from the placenta uniformly showed a significant association with CAM (9,10). The stimulatory effect of *Ureaplasma* spp. on cytokine release, such as tumor necrosis factor-α, IL-8, and IL-6, has been confirmed *in vitro* (20–22). Multiple-banded antigen and other lipoproteins from *Ureaplasma* spp. were found to activate nuclear factor kappaB through Toll-like receptor (TLR) 1, TLR2, and TLR6 and induce tumor necrosis factor-α in mouse peritoneal macrophages (23), indicating that not only viable *Ureaplasma* spp. but its lipoproteins cause an excessive immune response *in utero*. Umbilical vasculitis and chorionic plate inflammation might be caused by these antigenic components from local infection. To confirm the association of *Ureaplasma* spp. with CAM, we conducted *Ureaplasma* spp. cultivation as a prospective cohort study of 151 preterm placentas. Furthermore, immunohistochemical, PCR, and FISH analyses were performed in the infected placentas to confirm the microbial localizations.

We examined the incidence of *Ureaplasma* spp. colonization in the placenta by using the culture method. Studies involving various clinical specimens have shown PCR to be more sensitive than conventional culture methods (24–26). However, given the high sensitivity of PCR, a false positive result for a particular organism is more likely to occur than when culture methods are used, principally because of intersample contamination. Furthermore, PCR detects nonviable organisms as well as viable organisms, and does not enable further analysis using isolated microorganisms. On the other hand, culture for detection of *Ureaplasma* spp. is expensive and requires specialized media and expertise that are not widely available. We therefore established a culture method for the detection of *Ureaplasma* spp. by modification of the method of Shepard and Lunceford (27) Shepard and Combs (27,28), which uses materials that are all commercially available and easily prepared just before use.

The relation between infection and preterm delivery is not consistent throughout gestation. Infection is rare in late preterm delivery but is present in most cases in which birth occurs at <30 wk (29,30). Previous evidence suggests that intrauterine infection may occur early in pregnancy. For example, *Ureaplasma* spp. has been detected in some samples of amniotic fluid obtained in routine chromosomal analysis at 15–18 wk of gestation. In most of these women, delivery was around 24 wk (31–33). Meanwhile, Perni *et al.* (34) reported that in 179 asymptomatic women who received amniocentesis at midtrimester, all women with preterm PROM (5/5) tested positive for either *Ureaplasma* spp. or *Mycoplasma hominis* as opposed to none of the women (0/5) with spontaneous preterm birth. In this study, we showed that the incidence of *Ureaplasma* spp. colonization in preterm placentas was statistically higher than that in term placentas ($p < 0.05$) and that the incidence during the second trimester was higher than that during early in the third trimester ($p = 0.05$). We suggest that *Ureaplasma* infection-related CAM might be the leading cause of preterm delivery during the second trimester. One possible explanation for these gestational age-related changes in the cause of preterm delivery is that the intrauterine immune system during the second trimester might be susceptible to weakly pathogenic microorganisms such as *Ureaplasma* spp., and the hormonal immune response or cytokine production necessary to initiate labor is easily activated. In our study, the culture positive for other microorganism (Table 2) was lower (13/151; 8.6%) than those in previous reports (35,36). This discrepancy might be caused by 1) the treatment for inflammation and/or infection by bacteriocidal agents, which reduced the colonization in placentas, and 2) the samples, which we used was the placental surface swab but not by the homogenized tissues.

Although the association between *Ureaplasma* spp. colonization and CAM has already been identified, details of the placental features remain unclear. We therefore examined in detail all placentas delivered at <32 wk of gestation and investigated the presence of features specific to *Ureaplasma* spp. colonization. In the CAM placentas, polymorphonuclear leukocytes first accumulate in the intervillous space immediately below the chorionic plate, which forms the roof of this space. The inflammatory cells in the roof of the intervillous space later extend upward into the chorionic plate and reach the amnion. At this stage, the inflammatory cellular response is purely maternal in origin, the leukocytes being derived from maternal blood in the intervillous space (15). According to this migration process, we can simply imagine that this accumulation is composed of one layer of inflammatory cells extending toward the amniotic cavity. However, we found a characteristic pathologic feature named the amniotic bistratified pattern in which leukocytes did not infiltrate in one layer but in two layers. This pathologic pattern was significantly more frequent in placentas colonized with *Ureaplasma* spp. than that in others. Therefore, this amniotic bistratified pattern might be a specific placental feature of intrauterine *Ureaplasma* infection.

To explain how this infiltration pattern develops, we first hypothesized that inflammatory cells in the subchorionic space are derived from maternal sources, but those in the amnion are derived from fetal sources. However, we showed that neutrophils in both the subchorionic space and amnion were maternal cells. On the contrary, infiltrated neutrophils in the umbilical vein were of fetal origin (Fig. 3*D* and *G*). Earlier findings might be due to the differences in production and response between maternal and fetal neutrophils. Immature newborn infants frequently become neutropenic (37) in their response to bacterial sepsis. However, adults develop a sustained neutrophil leukocytosis by releasing preformed neutrophils from the marrow storage pool into circulation and increasing proliferation by recruiting a great number of committed granulocyte progenitors into the cell cycle. Furthermore, the most consistently observed functional abnormality of neonatal neutrophils is reduced chemotaxis. In most assays, neutrophils from newborn infants migrate at about half the speed of adult cells (38). Neonatal neutrophils display less interaction with endothelial monolayers in conditions of flow than adult cells. Rolling adhesion is diminished, and fewer cells attach to activated endothelium and migrate to the subendothelial tissue (39). These reports suggest that maternal neutrophils react predominantly at the fetomaternal interface; on the contrary, fetal neutrophils possess the immunologic reaction at least in the fetal side.

Next, we hypothesized that the development of this pattern might be involved in the distribution of genomic DNA and protein from *Ureaplasma* spp. as well as its receptor sulfoglycolipid in the placenta. There are no previous reports of their distribution, but we demonstrated that the urease structural gene and protein of *Ureaplasma* spp. were distributed in the amnion where maternal neutrophils accumulated and that their receptor sulfoglycolipid was also present in the amnion. Therefore, we suggest that this characteristic pattern of maternal neutrophil infiltration in the amnion might be associated with the distribution of *Ureaplasma* spp. and its receptor in the placenta.

In conclusion, our study indicates the following: 1) ~40–50% of preterm placentas delivered at <32 wk of gestation are culture positive for *Ureaplasma* spp.; 2) placentas colonized with *Ureaplasma* spp. show CAM significantly more frequently than others; 3) positivity of cultures for *Ureaplasma* spp. is an independent risk factor for CAM; 4) positivity of cultures for *Ureaplasma* spp. is also associated with the severity of CAM; and 5) the amniotic bistratified pattern in CAM might be a specific placental feature for intrauterine *Ureaplasma* infection.

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