

Pulmonary *Ureaplasma urealyticum* Is Associated with the Development of Acute Lung Inflammation and Chronic Lung Disease in Preterm Infants

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ABSTRACT

Previously, we have reported marked pulmonary inflammation in infants who develop chronic lung disease of prematurity. We revisited these infants who did not have clinical or laboratory evidence of infection and searched for *Ureaplasma urealyticum*, group B streptococci, and other microbes by reverse transcription-PCR performed on RNA extracted from 93 bronchoalveolar lavage samples. From infants ventilated for respiratory distress syndrome, 6 (gestation, 28 wk; birthweight, 880 g) were positive for *U. urealyticum* and 11 (25 wk, 800 g) were negative. Five (83%) positive and four (36%) negative infants developed chronic lung disease. Each infant was colonized with either biovar 1 or biovar 2 but not both. *U. urealyticum* was very weakly detectable in two infants on d 1 but was detected in five of six infants at d 10. Furthermore, pulmonary neutrophils, alveolar macrophages, soluble intercellular adhesion molecule-1,

and IL-1 β on d 10 and IL-6 and IL-8 at d 1 were significantly increased in the positive group. A variety of organisms were identified in six samples between 14 and 21 d of age, but all samples were negative for group B streptococci. Our data suggest that *U. urealyticum* colonization is associated with the development of pulmonary inflammation in infants who subsequently develop chronic lung disease. (*Pediatr Res* 55: 61–68, 2004)

Abbreviations

BAL, bronchoalveolar lavage fluid
CLD, chronic lung disease of prematurity
CRP, C reactive protein
ICAM, intercellular adhesion molecule
RDS, respiratory distress syndrome
RT-PCR, reverse transcription PCR

CLD remains a common respiratory disorder of preterm infants. Despite significant advances with therapeutic modalities, including the introduction of surfactant, various modes of mechanical ventilation and regular use of antenatal corticosteroids, morbidity and mortality remain high. Many risk factors have been identified and these include mechanical ventilation, oxygen therapy, patent ductus arteriosus, and nosocomial infection. An area that has recently received much attention is antenatal infection (1, 2). Even before the infant is delivered, an inflammatory response has been noted and this inflammation appears to be associated with the development of respiratory disease in the newborn infant (3, 4). One candidate for initiating this inflammation is the mycoplasma *Ureaplasma urealyticum* (5, 6). This organism has been isolated from the lungs of infants who have developed CLD, but whether *U.*

urealyticum causes acute lung injury in preterm infants or whether the association is with the degree of prematurity remains controversial (7). One approach to confirming or refuting this association is to study the link between the pulmonary inflammatory response, which has now been regularly reported in infants who develop CLD (8–11), and the presence of *U. urealyticum* in the lungs of infants who develop CLD.

Our previous data have demonstrated that proinflammatory cytokines IL-1 and IL-6, the potent neutrophil chemoattractant IL-8, and soluble adhesion molecule ICAM-1, as well as inflammatory cells, namely neutrophils, are increased in the lungs of infants who subsequently develop CLD (8, 9). These findings have been confirmed by many others (10, 11), and it is now generally accepted that pulmonary inflammation is a risk factor for the development of CLD (12). The exact injurious factors that trigger and maintain the inflammatory response remain elusive. Postnatal factors such as mechanical ventilation and oxygen therapy are likely candidates and antenatal infection may also be a factor (1, 2). In our previous

Received February 10, 2003; accepted May 30, 2003.

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DOI: 10.1203/01.PDR.0000100757.38675.50

studies, we had excluded any infants with evidence of infection that included prolonged rupture of membranes, an increase in peripheral blood neutrophils, a left shift of neutrophils, an increase in CRP, or positive blood or endotracheal cultures (8, 9). We revisited these samples and data to determine whether *U. urealyticum*, identified using RT-PCR and specific primers to the urease gene (13), was associated with an enhanced pulmonary inflammatory response and also whether the presence of this organism resulted in increased development of CLD. Because *U. urealyticum* can be grouped into two clusters, biovar 1 and biovar 2 (14), we also determined the pattern of colonization in each infant positive for *U. urealyticum*. As group B streptococci is associated with respiratory disease in newborn infants, we used specific primers for this organism to determine its presence in lung lavage samples. In addition, we also used universal primers for bacterial 16s ribosomal RNA (rRNA) genes, with subsequent cloning and sequencing techniques to identify the spectrum of organisms that colonized the respiratory tract of ventilated preterm infants who were at risk of developing CLD.

METHODS

In our previous studies, we only included those infants who developed RDS and who either recovered fully and were nursed in air and had normal chest radiographs by 28 d of age (RDS group) or who progressed to oxygen dependency and who had chest radiologic abnormalities beyond 28 d of age (CLD group) (8, 9). We also included four infants with muscular disorders but normal chest radiographs who received mechanical ventilation with inspired oxygen of <28% (control group). Infants with any evidence of infection, which included prolonged rupture of membranes, an increase in peripheral blood neutrophils, a left shift of neutrophils, systemic infection, an increase in CRP, or positive blood or endotracheal cultures, were excluded from the study. BAL was obtained as previously described (15, 16). Briefly, with the infant lying supine and head turned to the left, an FG5 catheter was inserted into the right lower lobe of the infant and two aliquots of 1 mL/kg of saline were instilled and, after two to four ventilator breaths, aspirated back. The cells were isolated by centrifugation and used to semi-quantitatively determine the expression of mRNA for IL-1 β , IL-6, and IL-8 (9). The supernatant was used to determine the concentration of agents of interest, namely IL-1 β , IL-6, IL-8, and ICAM-1, as previously reported (8, 9). A small aliquot of the lavage fluid was used to determine total cell and differential counts with a hemocytometer and a cytospin stained with Diff Quick (Dade Behring, Deerfield, IL, U.S.A.). The study was approved by the local research ethics committee, and informed consent was obtained from the parents before the lavage procedure.

Amplification by RT-PCR of BAL samples using universal bacterial 16s rRNA primers. The cDNA from the previous study (9) were used to amplify bacterial 16s rRNA genes. Sequences were amplified by PCR using the primers FD1 (AGA GTT TGA TCC TGG CTC AG) and rP1 (ACG G(T/A/C)T ACC TTG TTA CGA CTT) at concentrations of 0.2 μ M in the presence of Taq DNA Polymerase (Abgene, Epsom,

Surrey, U.K.). Before addition of the cDNA template to the PCR mix, reaction mixtures were treated with DNase I to remove any contaminating bacterial DNA in a similar protocol to that described by Hilali *et al.* (17). One microliter of DNase (Sigma Chemical, Poole, Dorset, U.K.) solution at a concentration of 0.0625 Kunitz units/ μ L was added to each 50 μ L reaction and the tubes incubated at 37°C for 15 min followed by heating at 94°C for 50 min to inactivate the DNase. The cDNA template was then added to the PCR mixes including the primers described above and subjected to 95°C for 2 min followed by 30 cycles at 95°C for 30 s, 55°C for 40 s, and 72°C for 2 min followed by a 10-min extension cycle at 72°C.

After amplification by PCR, 1 μ L of the reaction product was re-amplified using the same protocol as above but substituting the rP1 primer with rD2 (G(T/A)A TTA CCG CGG C(G/T)G CTG). The subsequent reaction products were separated by electrophoresis on 1.2% agarose gels stained with ethidium bromide and visualized using UV illumination. The PCR products were cut from the gel and purified using the QIAquick gel extraction system (QIAGEN, Dorking, Surrey, U.K.). The fragments were cloned into the pGEM-T Easy vector system (Promega UK Ltd., Southampton, U.K.) and sorted by restriction fragment length polymorphism (RFLP) analysis before DNA sequencing of unique clones. The closest relatives for the sequences obtained were determined by BLAST searches of databases posted at <http://www.ncbi.nlm.nih.gov/>.

Amplification by RT-PCR of BAL samples using specific primers to *U. urealyticum* and group B streptococci. To confirm the presence of intact cDNA in each sample, β -actin was amplified in each cDNA sample using primers previously described (9). Primers specific for the urease gene of *U. urealyticum* (U5—CAA TCT GCT CGT GAA GTA TTA C and U4—ACG ACG TCC ATA AGC AAC T), as described in Blanchard *et al.* (13), were used to identify the presence of *U. urealyticum* urease DNA in cDNA obtained from BAL cell pellets. Amplification by PCR with these primers resulted in a 428-bp product fragment of the components UreA and UreB of the *U. urealyticum* urease complex. *U. urealyticum* biovars were differentiated using primers UMS 125 (GTA TTT GCA ATC TTT ATA TGT TTT CG) and UMA 226 (CAG CTG ATG TAA GTG CAG CAT TAA ATT C), which resulted in a 403-bp amplified product for biovar 1 and a 448-bp product for biovar 2 (14). Primers specific for group B streptococci, Sag59 (TTT CAC CAG CTG TAT TAG AAG TA) and Sag190 (GTT CCC TGA ACA TTA TCT TTG AT), as described by Ke *et al.* (18), were used to identify the presence of this organism in cell pellets obtained by BAL. Amplification by PCR with these primers resulted in a 153-bp fragment of the group B streptococci cAMP gene.

For each gene of interest, amplification was carried out using Taq DNA Polymerase (Abgene) at 95°C for 2 min followed by 30 cycles of 95°C for 30 s, 55°C for 40 s, and 72°C for 1 min followed by a 10-min extension cycle at 72°C. The reaction products were separated by agarose gel electrophoresis and the presence or absence of bands was identified by examination of the gels under UV illumination.

Table 1. Pattern of presence of *U. urealyticum* in bronchoalveolar lavage fluid samples from ventilated preterm infants with at least one positive sample

Baby No.	d 1	d 4	d 7	d 10	d 14	d 17	d 21	d 25	d 28	d 31	d 35	d 38
1		–	–	+	+	–	+	–		–		
2	±	+	+	+	+	+	+	–	–	–	–	–
3	–	–	+	+	–							
4	±	–	+	+	+	+	+	–	–			
5	–	+	–	–	–	+	–	–		–	–	
6	–	–		+								

Statistical analysis. The medians for the birth weight and gestation are given. The cellular and cytokine concentrations are given as mean ± SEM and the nonparametric test Mann-Whitney *U* test was used to compare the results obtained for the groups that were positive and negative for *U. urealyticum*. Fishers exact test was used to compare the infants with or without pulmonary *U. urealyticum* with the development or not of CLD.

RESULTS

Patient characteristics. Ninety-three BAL samples were obtained from 21 infants. Seventeen of these developed respiratory failure due to RDS and, of these, nine progressed to develop CLD, *i.e.* oxygen dependency, at 28 d of age. Four infants were ventilated for nonrespiratory reasons. Significant differences were noted between the RDS [birth weight, 820 g (range, 570–970); gestation, 25 wk (range, 24–29)] and CLD [birth weight, 990 g (780–2130); gestation, 28 wk (27–33)] groups for both gestation (*p* < 0.01) and birth weight (*p* < 0.05).

Detection of *U. urealyticum* urease gene in BAL samples. Using specific primers for *U. urealyticum*, we were able to identify six infants with at least one positive sample (Table 1). In all infants but one, *U. urealyticum* appeared to be present at 10 d of age. In contrast, all samples at d 1 (and d 4 sample for infant 2) were negative for this organism. We therefore subjected these d 1 samples (and d 4 sample for infant 2) from the positive group, together with all d 1 samples in the negative group, to two rounds of PCR to determine whether there was a low microbial load (*i.e.* the product from the first PCR run of 30 cycles was re-amplified again with a further 30 cycles using the specific primers for the urease gene of *U. urealyticum*). None of the infants in the negative had *U. urealyticum* detected in the d 1 samples, but two infants in the positive group had only very weakly detectable signals at d 1 (Table 1). None of the control infants were positive for *U. urealyticum*. Four infants in the positive group had all samples positive for biovar 1 and two were positive for biovar 2. All samples in each infant were colonized with either biovar 1 or biovar 2 but not both. We reclassified the 17 infants in the CLD and the RDS groups according to whether the infants had at least one positive sample for *U. urealyticum*; the patient data are shown in Table 2 and the cellular and cytokine data are shown in Figures 1–4.

Although the infants in the *U. urealyticum* group were heavier (880 g; range, 690–2310 g) when compared with the negative group (800 g; range, 570–910 g), this difference was

Table 2. Patient characteristics of infants in the *U. urealyticum* positive, negative, and control groups

	<i>Ureaplasma</i> positive	<i>Ureaplasma</i> negative	Control
Number	6	11	4
Gestation (wk)	28 (24–33)	25 (24–27)	38 (31–40)
Birth weight (g)	880 (690–2310)	800 (570–910)	2690 (1380–3240)
Male:female	4:2	4:7	3:1
CLD	5/6 (83%)	4/11 (36%)	0/4 (0%)
Antenatal dexamethasone	3/6 (50%)	7/11 (64%)	1/4 (25%)
Surfactant	6/6 (100%)	10/11 (91%)	0/4 (0%)

Median (range).

not statistically significant. However, the infants in the positive group were significantly more mature (28 wk; range, 24–33 wk) than those in the negative group (25 wk; range, 24–27 wk, *p* < 0.05). Five of the 6 (83%) infants positive for *U. urealyticum* developed CLD, but only 4 out of 11 (36%) in the *U. urealyticum*–negative group developed CLD. The infant who did not develop CLD in the positive group had severe respiratory failure and survived his twin brother, who died from severe RDS but had no clinical or laboratory evidence of infection. There was a significant association between the presence of *U. urealyticum* and the development of CLD (*p* < 0.05).

Cellular and cytokine data for infants positive and negative for *U. urealyticum*. The total cell counts in BAL fluid from the infants in the *U. urealyticum*–positive group were between 3.3 and 3.4 × 10⁵/mL between d 1 and 7 of age before increasing to 5.5 × 10⁵/mL on d 10 of age (Fig. 1A). Thereafter, the total cell counts decreased to 1.6 × 10⁵/mL before reaching a maximum of 14.0 × 10⁵/mL at d 17 of age. In the *U. urealyticum*–negative group, the total cells counts of 3.3 × 10⁵/mL on d 1 decreased to 0.9 × 10⁵/mL by d 7 before gradually increasing to 10.1 × 10⁵/mL by d 17. For neutrophil counts in BAL fluid, the counts were similar between the two groups during the first 7 d of life, but the counts were significantly higher in the *U. urealyticum*–positive group at d 10. The counts increased in both groups to a maximum on d 17 of age (Fig. 1B). A marked difference was noted between the two groups for alveolar macrophages, which remained <0.8 × 10⁵/mL in the *U. urealyticum*–negative group but increased from 1.0 × 10⁵/mL on d 1 to 2.6 × 10⁵/mL on d 4 before gradually declining to 0.2 × 10⁵/mL on d 14 of age (Fig. 1C). A further increase was noted on d 17 to 3.1 × 10⁵/mL.

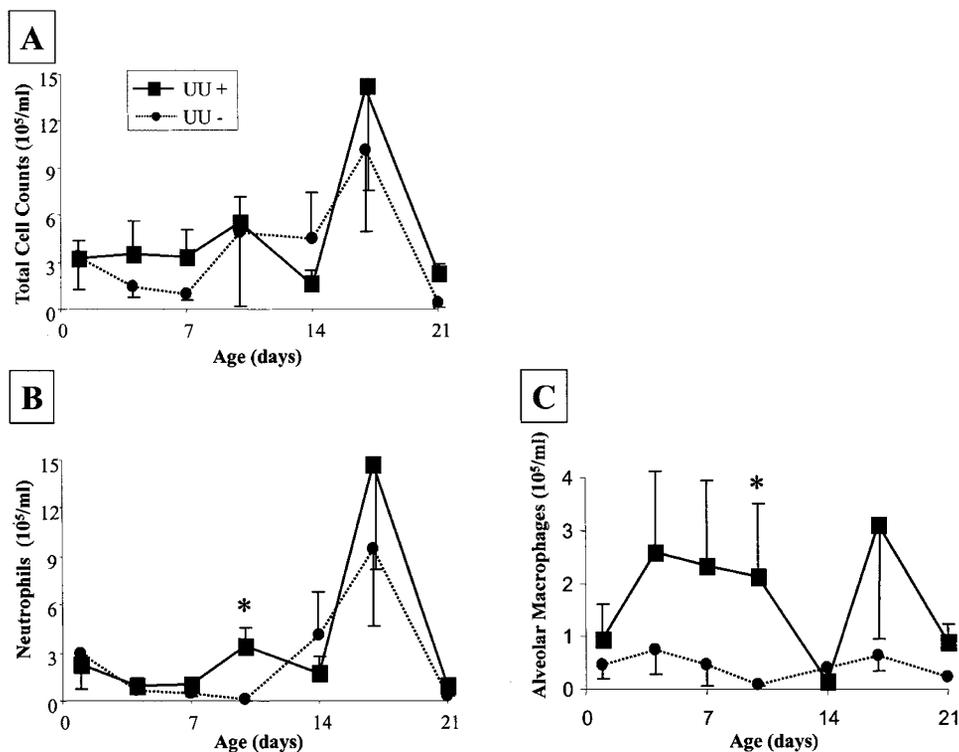


Figure 1. (A) Absolute total cell, (B) neutrophil, and (C) alveolar macrophage counts in BAL obtained from preterm infants requiring mechanical ventilation for RDS. The infants were divided according to whether they were positive (solid lines) or negative (dashed lines) for *U. urealyticum* by RT-PCR. * $p < 0.05$.

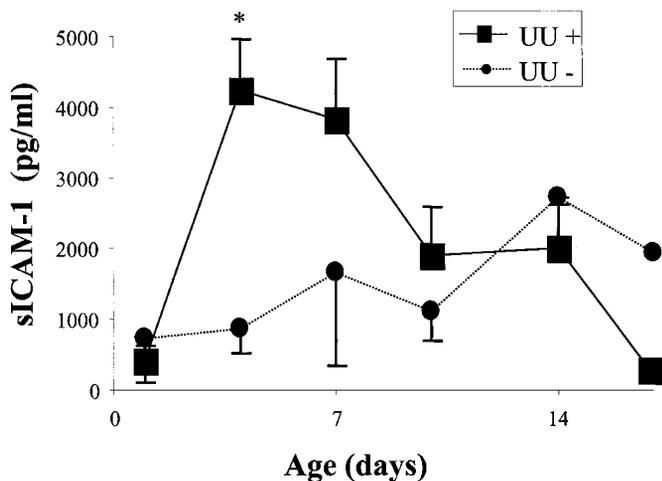


Figure 2. Soluble ICAM-1 concentration in BAL obtained from preterm infants requiring mechanical ventilation for RDS. The infants were divided according to whether they were positive (solid lines) or negative (dashed lines) for *U. urealyticum* by RT-PCR. * $p < 0.05$.

A similar pattern to alveolar macrophages was observed for sICAM-1 in BAL samples in that its concentration increased from 430 pg/mL on d 1 to 4240 pg/mL on d 4 before declining gradually to 217 pg/mL by d 17 in the *U. urealyticum*-positive group (Fig. 2). In the *U. urealyticum*-negative group, the concentration of sICAM-1 in BAL samples increased gradually from 727 pg/mL at d 1 to a maximum of 2741 pg/mL on d 14 of age.

For IL-1 β in BAL samples, the pattern of change was broadly similar in both groups in that a low concentration on d 1 increased in both groups to reach a maximum of 1653 pg/mL

at d 14 in the *U. urealyticum*-negative group and 2394 pg/mL on d 17 in the *U. urealyticum*-positive group (Fig. 3A). However, the concentrations appeared to be greater in the latter group, where significant increases were noted on d 7. The expression of IL-1 β mRNA increased in the *U. urealyticum*-positive group from 0.6 (ratio with β -actin) on d 1 to a maximum of 1.2 on d 10 of age (Fig. 3B). In the negative group, the IL-1 β / β -actin ratio was 1.0 at d 1 but remained <0.5 thereafter. Significant increases were noted in the positive group at 7 d of age.

The concentration of IL-6 in lung lavage fluid at d 1 in the *U. urealyticum*-positive group was significantly increased when compared with the values obtained for the *U. urealyticum*-negative group (Fig. 3C). Its concentration decreased to 1339 pg/mL over the next 3 wk. In contrast, in the *U. urealyticum*-negative group, the concentration of IL-6 in lung lavage fluid remained <1286 pg/mL except for an increase at d 14 to 2394 pg/mL. The IL-6/ β -actin mRNA ratios increased from 0.4 in the *U. urealyticum*-positive group to a maximum of 1.1 on d 10 before declining to 0.4 on d 17 of age (Fig. 3D). In the *U. urealyticum*-negative group, the IL-6/ β -actin mRNA ratio was <0.4 except at d 10 (1.5) and 17 (0.8).

In the *U. urealyticum*-positive group, the concentration of IL-8 in BAL fluid was significantly increased at d 1 (13.1 ng/mL versus 2.2 ng/mL) (Fig. 4A). This decreased to 6.7 ng/mL by d 14 before increasing to 11.3 ng/mL by d 21. In the negative group, the concentration of lung lavage fluid IL-8 was 2.2 ng/mL on d 1 and this increased to 27.0 ng/mL on d 10 before declining to 2.1 ng/mL on d 21. The IL-8/ β -actin mRNA ratio was low (0.3) on d 1 in the *U. urealyticum*-positive group but increased to reach a maximum at d 7 (1.8)

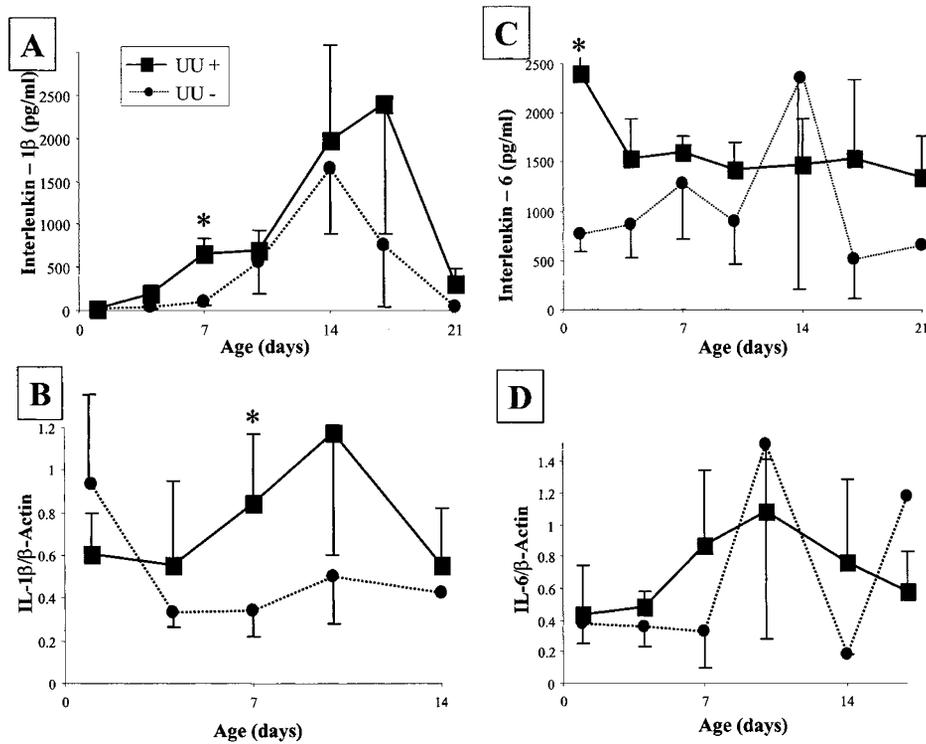


Figure 3. (A) Concentration of IL-1 β , (B) the ratio of IL-1 β mRNA against β -actin mRNA, (C) concentration of IL-6, and (D) the ratio of IL-6 mRNA against β -actin mRNA in BAL obtained from preterm infants requiring mechanical ventilation for RDS. The infants were divided according to whether they were positive (solid lines) or negative (dashed lines) for *U. urealyticum* by RT-PCR. * $p < 0.05$.

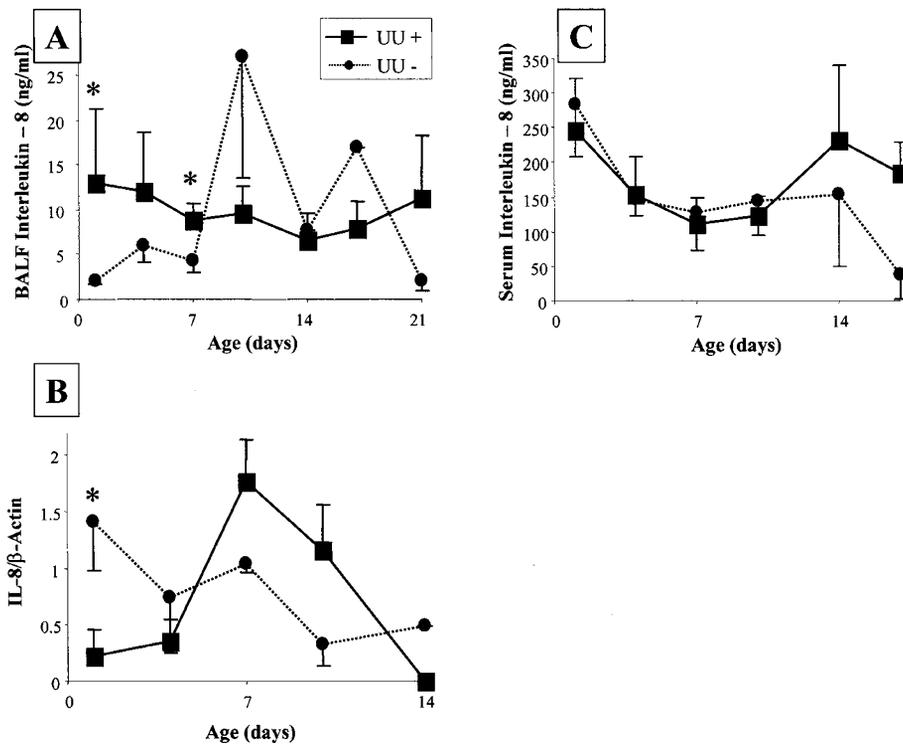


Figure 4. (A) Concentration of IL-8 in BAL, (B) ratio of IL-8 mRNA against β -actin mRNA in cells obtained by BAL, and (C) concentration of IL-8 in serum. The infants were divided according to whether they were positive (solid lines) or negative (dashed lines) for *U. urealyticum* by RT-PCR. * $p < 0.05$.

before declining to undetectable levels by d 14 (Fig. 4B). By contrast, in the *U. urealyticum*-negative group, the IL-8/ β -actin ratio was increased to 1.4 at d 1 and this decreased to 0.5

on d 14. The concentration of serum IL-8 was similar in both groups during the first 10 d of life but appeared to remain elevated in the *U. urealyticum*-positive group at d 14 and 17,

although the differences were not significant. The concentration of serum IL-8 in both groups was an order of magnitude less than that noted in BAL samples (Fig. 4C).

To understand why not all samples in individual infants were positive for *U. urealyticum*, we hypothesized that BAL samples positive for *U. urealyticum* would be associated with increased alveolar macrophages and pro-inflammatory cytokines when compared with BAL samples that were negative for *U. urealyticum*. Alveolar macrophages appeared to be increased in individual BAL samples that were positive for *U. urealyticum* ($p < 0.05$) but neutrophils were not (NS). In addition, the pro-inflammatory agents IL-6 ($p < 0.01$) and IL-1 ($p < 0.001$) as well as ICAM-1 ($p < 0.05$) were all increased in BAL from the positive group but IL-8 (NS) was not.

Presence of bacterial ribosomal rRNA genes in BAL cells. When we used RT-PCR to detect the presence of 16S rRNA genes using universal bacterial primers, only six samples between the ages of 14 and 21 d were found to be positive (Fig. 5). Subsequent cloning and DNA sequencing demonstrated a wide variety of organisms colonizing the respiratory tract of these infants, including the recovery of 16S rRNA genes of *U. urealyticum*. Other 16S rRNA genes included, interestingly, that of *Stenotrophomonas maltophilia*, an organism implicated in cystic fibrosis. *Brevundimonas*, *Sphingomonas*, and *Pseudomonas* spp. all are occasionally isolated from clinical specimens, often of doubtful clinical significance. The ubiquitous *Staphylococcus aureus* may be a contaminant and *Gemella haemolysans* is a commensal of the gastrointestinal tract (Table 3). It was interesting to note that the infants were chronically

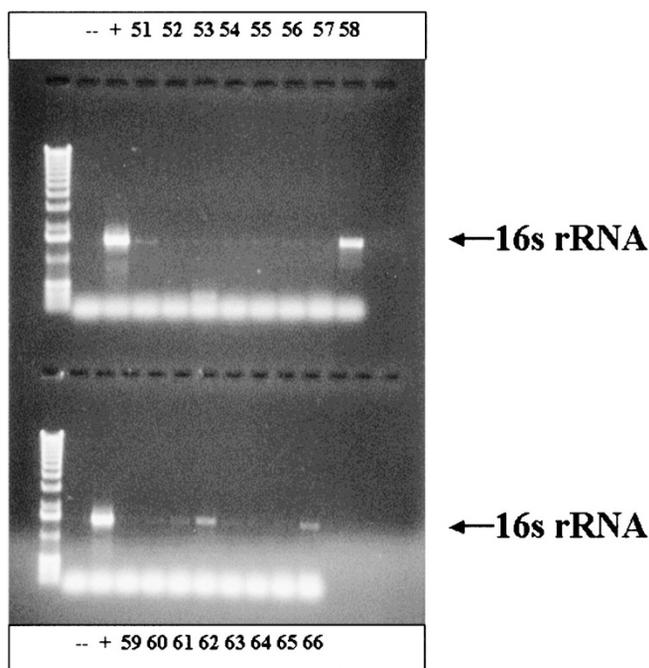


Figure 5. Total RNA was extracted from cells and other material obtained by bronchoalveolar lavage of ventilated preterm infants and reverse transcribed. The resulting cDNA was amplified by PCR using universal primers to bacterial 16S ribosomal RNA and the amplified products separated on an ethidium bromide stained gel. The amplified products were cloned and sorted by RFLP before DNA sequencing of unique clones. The closest microbial relatives were determined against published databases. The results are shown in Table 3.

Table 3. Detection of a variety of organisms by cloning and sequencing the fragments of 16 rRNA amplified by RT-PCR

Baby T	50% <i>U. urealyticum</i> 30% <i>Shigella sonnei</i> or <i>Escherichia coli</i> 10% <i>Brevundimonas</i> sp. 10% unknown
Baby T	30% <i>Brevundimonas</i> sp. 30% <i>Rathayibacter festuci</i> 40% unknown
Baby W	<i>Staphylococci epidermidis</i>
Baby G	30% <i>Delftia</i> sp. 30% <i>Brevundimonas</i> sp. 20% <i>Stenotrophomonas maltophilia</i> 10% <i>U. urealyticum</i> 10% unknown
Baby G	<i>Gemella haemolysans</i>
Baby L	50% <i>Sphingomonas</i> sp. 30% <i>Stenotrophomonas maltophilia</i> 10% <i>Staphylococci epidermidis</i> 10% <i>Pseudomonas putida</i>

ventilated and did not have any clinical differences from those who were not colonized. As beta-hemolytic group B streptococci are often associated with respiratory disease, particularly in term and near-term infants, we used specific primers to this organism to identify its presence in lung lavage samples. All our infants receiving mechanical ventilation were treated with penicillin and gentamicin and none of the samples were positive for this organism by RT-PCR (data not shown).

DISCUSSION

In this study, we have identified the presence of *U. urealyticum* by RT-PCR in infants who did not have clinical or laboratory evidence of infection, although culture for this organism was not routine. Almost all of these infants developed CLD and had a marked inflammatory response in their lungs. Furthermore, the *U. urealyticum* was identified in almost all these infants at 10 d of age but, despite the use of very sensitive techniques, d 1 samples were weakly positive for this organism in only two infants after two rounds of RT-PCR. Furthermore, every sample from each infant was colonized with either biovar 1 or biovar 2 and no infant was colonized with both biovars. We were unable to demonstrate any differences between those colonized with either biovar. Interestingly, the infants who were positive for *U. urealyticum* were more mature than those who were negative for this microbe. Several of the infants were colonized with a variety of organisms at 14–21 d of age, but, using specific primers, group B streptococci, a cause of severe respiratory failure in newborn infants, were not detected in any of the infants in the study.

U. urealyticum has previously been shown to be associated with the development of CLD, but its association with prematurity leaves open the question as to whether this microbe causes acute lung injury or is simply an innocent bystander (7). In this study, *U. urealyticum* was associated with a more mature group of infants who had a greater chance of developing CLD than the more immature infants who did not have any evidence of infection. It should be remembered that only infants who did not have clinical or laboratory evidence of

infection were studied. Furthermore, the presence of this organism was associated with a marked pulmonary inflammatory response when compared with infants who were not colonized with *U. urealyticum*. Although mechanical ventilation and oxygen are also risk factors for the development of pulmonary inflammation, the ventilatory parameters were similar in both groups, thus it is likely that these factors were responsible for the different patterns observed.

We and others have previously associated the development of CLD with an airway neutrophilia (8, 19), but in the *U. urealyticum*-positive group the more impressive increase was of alveolar macrophages (Fig. 1C), although an airway neutrophilia was also noted at 10 d of age. Coalson's group has previously noted an increase in alveolar macrophages in a baboon model of prematurity and infection with *U. urealyticum* (20). Our data are also compatible with those reported in a murine model of *U. urealyticum* infection in which an initial airway neutrophilia was followed by increased airway macrophages (21). Studies of postmortem lungs by Viscardi's group showed that alveolar macrophages predominated when the infants were colonized with *U. Urealyticum* but with airway neutrophilia when they were not (22).

Similarly, several authors have reported an increase in pro-inflammatory cytokines in infants who are colonized with *U. urealyticum* (23, 24). Our study adds further to this literature by suggesting that in infants who do not have clinical or laboratory evidence of sepsis, *U. urealyticum* must be considered as an important contributor to acute lung injury, as was demonstrated by an increase of pro-inflammatory cytokines, particularly IL-6 and IL-8, which were both increased at birth in these infants. Other pro-inflammatory agents, including neutrophils, sICAM, and IL-1 β , appeared to be increased at d 7–10 of age in the *U. urealyticum* positive group. Although there have been recent reports of a systemic fetal inflammatory response (25), our data suggests that there was no difference at birth in serum IL-8 in the two groups studied. Interestingly, the concentration of serum IL-8 was at least an order of magnitude lower than in lung lavage fluid, suggesting a localized inflammatory response within the lungs of infants who progress to develop CLD.

We were surprised by the finding of *U. urealyticum* at d 10 of age in almost all infants and by its absence on d 1 in all infants after one round of PCR. We attempted to identify whether this organism was present on d 1 by using multiple rounds of PCR but were only able to weakly detect the presence of this organism in two samples from the positive group at d 1. Our current understanding suggests that *U. urealyticum* is acquired through the genital tract, and yet the d 1 samples were either negative or only very weakly positive in two infants. By the nature of the study, samples collected from any infants with clear evidence of infection would have been excluded, and it is likely that the microbial load at birth is very low, but our technique is exquisitely sensitive to detect even one individual organism. Alternatively, technical limitations, e.g. of the lung lavage procedure or of the organisms viability, may affect the results. It may be that *U. urealyticum* may be acquired antenatally or postnatally (26). A recent study by Castro-Alcaraz *et al.* (27) reported three patterns of *U. urea-*

lyticum colonization of preterm infants: persistent, early transient, and late transient. Those associated with persistent colonization, as in our study, were associated with the development of CLD. Additional work is clearly required to determine whether acquisition of the organism is prenatal or postnatal.

Interestingly, *U. urealyticum* was not present in all samples from individual infants in the positive group. The sampling technique may account for this observation, as the presence of *U. urealyticum* is unlikely to be uniformly distributed throughout the lung. Alternatively, the presence of this organism appears to be related to the presence of alveolar macrophages. We thus compared all samples positive for *U. urealyticum* with the negative samples and were able to demonstrate a strong association between positive samples and alveolar macrophages as well as IL-1, IL-6, and sICAM-1. It may be that where the alveolar macrophages were present in numbers, the likelihood of detecting *U. urealyticum* was also increased. The data illustrate that single samples may miss the presence of *U. urealyticum*. It is difficult to explain why all samples beyond 21 d of age were negative (Table 1), but this may be related to sampling technique or decreased presence of alveolar macrophages, or due to the infants resolving their infection. This observation is unlikely to be due to antibiotic treatment as none of the infants were treated with erythromycin.

Although our study is limited by a number of factors, we ensured that sufficient cDNA was available for all samples by detecting the presence of β -actin by RT-PCR. At the time the samples were collected, culture for *U. urealyticum* or other mycoplasmas was not routine. Indeed, only a limited number of centers, chiefly interested in research, routinely culture for mycoplasmas. Clearly, our data suggest that routine culture may be indicated in infants who are at risk of developing chronic respiratory failure and are likely to remain oxygen dependent for prolonged periods. Furthermore, typing of the *U. urealyticum* may be necessary as it has implications for the organism's resistance to antibiotics and hence the appropriate use of antibiotics (14). Many infants born prematurely to mothers with chorioamnionitis appear not to develop RDS (6) and thus are unlikely to feature in this study. These infants are likely to have a greater prevalence of infection with *U. urealyticum* and other microbes, as would women with prolonged rupture of membranes or those with fetal inflammatory response (28). In our study, the focus was specifically on infants who did not have any clinical or laboratory evidence of infection at birth and thus would have excluded such infants born to mothers with chorioamnionitis, prolonged rupture of membranes, or fetal inflammatory response. Our results suggest that *U. urealyticum* may be prevalent more often than previously thought.

There appears to be a discrepancy of inflammatory responses as suggested by the increased IL-6 and IL-8 at birth and a later increase at d 10 of neutrophils, alveolar macrophages, sICAM, and IL-1 β (Fig. 1–4). However, there are a large number of risk factors that may lead to the development of CLD in preterm infants. Until recently, most research has focused on postnatal factors, particularly mechanical ventilation and oxygen toxicity (29). However, antenatal factors, including uterine

infection (1, 2), may initiate the inflammatory process in the lungs of infants who are destined to develop CLD (3, 4). It is likely that in the infants who were positive for *U. urealyticum* the pulmonary inflammatory process was initiated antenatally and maintained postnatally by the risk factors of CLD. It has been reported that the lung injury is exacerbated by oxygen if the lung is already infected or colonized with *U. urealyticum* (30). Thus, despite similar ventilatory requirements in both groups, the positive group was subjected to greater lung injury due to the presence of *U. urealyticum*.

Only a small number of samples and infants were positive for the presence of 16 rRNA. All samples had been obtained from infants who had been chronically ventilated for respiratory failure and the positive samples were from otherwise well infants at 14–21 d of age, suggesting that chronic ventilation is associated with a wide variety of bacterial types of doubtful clinical significance, although the presence of the organism *Stenotrophomonas maltophilia* is interesting in view of its association with cystic fibrosis. Encouragingly, we did not detect group B streptococci in any of samples. This may be due to all the infants being treated with a combination of penicillin and gentamicin.

Antenatal infection has received much attention recently as a possible cause of respiratory disease in newborn infants. Our data suggest that one such microbe, namely *U. urealyticum* may at least in part be responsible for the pulmonary inflammation that has been consistently reported in infants who develop CLD (8–12). Whether this inflammatory process can be prevented by the use of appropriate antibiotics needs to be investigated in greater detail. It is clear from this study that the newer, more sensitive and accurate techniques have a role to play in understanding mechanisms of acute lung injury in newborn infants.

Acknowledgments. The authors thank the parents of the infants in this study and staff at the Hammersmith Hospital.

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