THE YALE JOURNAL OF BIOLOGY AND MEDICINE 56 (1983), 565-572

Serological Evidence of *Ureaplasma urealyticum* Infection in Neonatal Respiratory Disease

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Received January 4, 1983

Since up to 80 percent of pregnant women and 30 percent of neonates may be colonized with genital mycoplasmas, it is difficult to determine whether true infection occurs. The antibody responses to eight serotypes of *U. urealyticum* were assessed in mothers and infants in 21 cases of neonatal respiratory disease (RD) and 24 normal cases. Among the normal population of mothers and infants, a titer of ≥ 1:32 occurred in 0.25 percent (1/394). In mother-infant paired titers, a fourfold difference occurred in 2.6 percent (5/192). Among 54 RD neonates, 55.6 percent had a titer of ≥ 1:32 compared to only 4.2 percent of normal neonates ($p < .001$). Fourfold elevations in antibody titers of > 1:32 were observed in the neonate in 52.4 percent of RD cases compared to 0 perecnt of 24 normal pairs ($p < .001$) and in 28.6 percent of mothers of RD neonates compared to 0 percent in normal cases ($p = .013$). We observed that 43.3 percent of RD neonates with titers ≥ 1:32 died compared to 16.6 percent of RD neonates exhibiting no elevation of antibody response over the maternal level. Among the six who died, 66.7 percent of neonates and 16.7 percent of their mothers had elevated titers, compared to 33.3 percent of 15 surviving infants and 40.0 percent of their mothers. These elevated antibody responses strongly support the concept that *U. urealyticum* causes infection in the perinatal period in association with neonatal respiratory disease. Since the elevation in titers was detected close to delivery in many cases, the infection may occur *in utero*.

INTRODUCTION

Asymptomatic mycoplasma colonization occurs in 6–75 percent of adults and up to 80 percent of pregnant women. Newborns are colonized at birth in 15–33 percent of cases [1]. The presence of *Ureaplasma urealyticum* and/or *Mycoplasma hominis* in placentas is associated with chorioamnionitis, low birth weight, and a significantly higher rate of spontaneous abortion, prematurity, perinatal morbidity, and mortality [1–4]. Mycoplasmas have been isolated from amniotic fluid [1,5] and are associated with spontaneous abortion and congenital pneumonia [1–11].

Mycoplasmas from the genital tract have been associated with fetal pneumonia in spontaneous abortion and congenital pneumonia in neonates based on the isolation of the organism and histological evidence of inflammation in the lung [6–10]. In a recent case of congenital pneumonia based on isolation and immunospecific staining of the lung tissue, intrauterine infection was confirmed by the presence of an

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elevated antibody titer at birth and the presence of IgM specific antibody at 74 hours of age, suggesting infection began *in utero* [11]. Spontaneous abortion and "weak calf syndrome" (with respiratory distress) were also observed in bovines infected in pregnancy with *U. diversum* isolated from an aborted calf [12].

The possibility of intrauterine infection has been difficult to assess because isolation of the organism does not distinguish between intra-partum colonization and true infection. In this paper, serological data on infants born with respiratory disease support the concept that *U. urealyticum* causes lung disease in neonates of varying gestational ages.

MATERIALS AND METHODS

Patients

Antibody levels were determined on paired mother-infant sera from *(a)* 24 normal healthy deliveries taken at birth, and *(b)* 21 pairs plus 33 neonates who were born with respiratory disease (RD) of unknown etiology which was fatal or developed a protracted course. These neonates were born between 25–40 weeks of gestation with sera drawn one to 20 days after delivery.

Serology

The paired sera were assayed simultaneously by a modified metabolic inhibition test [13,14] for eight serotypes of *U. urealyticum.* The strains used were as follows: Serotype 1 (strain 7), 2 (strain 23), 3 (strain 27), 4 (strain 58), 5 (strain 354), 6 (strain Pi), 7 (strain Co), and 8 (strain T960-CX8). The strains were obtained from Dr. R. Purcell at the National Institutes of Health, Bethesda, MD. Trypticase soy broth [3] supplemented with 20 percent horse serum (K.C. Biologicals), 10 percent of fresh yeast extract (25 percent w/v), 10 percent guinea pig serum (Gibco), 0.1 percent urea, and 0.004 percent bromothymol blue was used. Each assay was done with 10^2 and 10^3 ccu/ml final concentration of ureaplasma as recommended by Lin [14]. The initial dilution of serum assayed was 1:8 since the organism and media were added at the same time prior to incubation. The titer was read when the control cups containing ureaplasma without patient sera had changed 0.5 to 0.75 pH units. Plates were read every 30 minutes once the color changes started. The endpoint was recorded as the highest dilution of serum which inhibited growth and held constant for two consecutive readings (at least 30 minutes). Occasionally, when control cups did not turn positive by day end, the plates were put at 30°C overnight. In most cases the titer was the same. The plate was re-incubated at 36°C and read throughout the day. This was essential since different serotypes grew at different rates. A pattern of inhibition of one dilution less for 10^3 ccu/ml from the reading at 10^2 ccu/ml was used as a guide to control for spotting (lack of growth) in some cups of the microtiter plates.

Statistical Methods

Comparisons of the frequency of elevated titers used Chi^2 or Fisher's exact test when expected frequencies were small. Means of mothers and neonates in the RD group were compared to normal mothers and neonates using a *t*-test and the logarithm of the antibody titers. Titers of $< 1:8$ were taken as 1:4 to calculate means. The means of the log titers have been converted back to the observed scale of dilutions for presentation in Table 2 and Fig. 1.

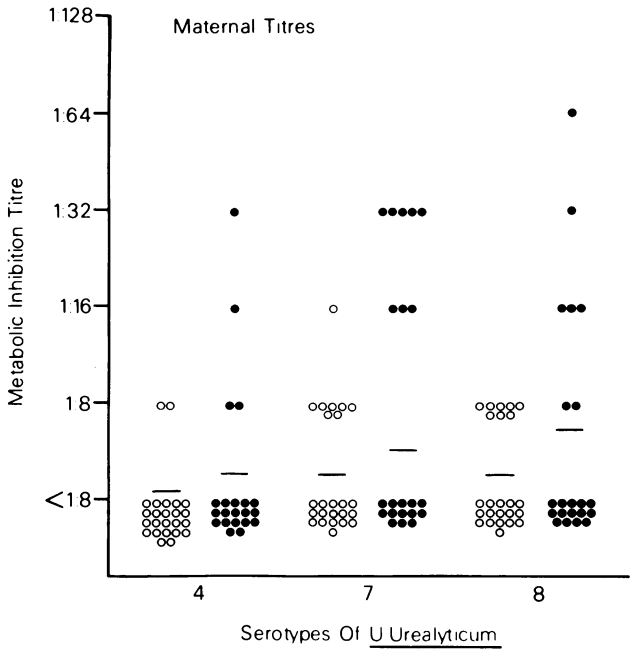

FIG. 1. Distribution of antibody responses to *U. urealyticum* serotypes *(a)* in normal neonates and their mothers (○) and *(b)* in those born with respiratory disease (●), The mean titers are indicated by the bars. p values are <0.05 (*) and <0.01 (**).

RESULTS

Comparison of Antibody Titers to U. urealyticum *in Normal Deliveries*

Antibody titers to eight serotypes were determined on 24 randomly selected mother-infant pairs with normal gestation, delivery, and healthy neonates. In the 192 paired MI tests, the mother's titer was equal to that of the neonate in 71.9 percent of the assays. A twofold difference either way was observed in 25.5 percent. A fourfold difference between the mother's and infant's titer to a given serotype was observed in only 2.6 percent of cases. We therefore considered a titer fourfold higher to be significantly elevated. Among the 394 titers obtained from normal mothers and infants, a value of $\geq 1{:}32$ was observed only once to one serotype (0.25 percent). This suggested that a titer $\geq 1{:}32$ was above the normal range for a healthy population.

Elevation of Antibody Levels to U. urealyticum *Serotypes*

Antibody levels were compared in 21 RD neonates born between 25–42 weeks of gestation and 24 normal neonates born at term and their mothers. A fourfold elevation in neonatal titer to at least one serotype was found in 47.6 percent of RD cases compared to 16.7 percent of normal pairs ($p = .056$). Mothers of RD neonates exhibited elevated titers over their infants at delivery in 33.3 percent of cases compared to 8.3 percent of mothers of normal neonates ($p = .084$). Among the normal pairs, the elevated titers were from $<1{:}8$ to 1:16 whereas in the RD pairs the titers ranged from $<1{:}8$ to 1:128. However, in cases where the maximum titer was $\geq 1{:}32$, 52.4 percent of RD neonates exhibited elevations compared to 0 percent among normal neonates ($p<.001$) and 28.6 percent of RD mothers had elevated titers compared to 0 percent of normal mothers ($p = .013$).

When the actual titers were compared, 57.1 percent (12/21) of RD neonates with a

TABLE 1
Neonatal Mortality in Relation to Elevated Titers to *U. urealyticum*

	Mean Gestational Age (weeks)	No.	Proportion of Deaths (%)
Infant Titer			
≥1:64	32.3	11	6 (54.6)
1:32	32.0	19	7 (36.8)
≤1:16	30.9	24	9 (37.5)
4× Above Maternal Titer			
To 1:64 or higher	37.0	1	1 (100)
To 1:32	33.5	4	1 (25)
To 1:16	29.5	4	1 (25)
No. 4× elevation	29.4	12	2 (17)

mean gestational age of 31.6 weeks had a titer ≥1:32 to at least one serotype of *U. urealyticum* compared to 4.2 percent of normal term neonates ($p<.001$). Among the mothers, 66.7 percent of those with RD neonates had at least one titer of ≥1:32 compared to 0 percent of the normal mothers ($p<.001$).

Mortality Rate with U. urealyticum *Infection*

To correlate the effect of *U. urealyticum* as a cause of severe disease, the mortality rate was assessed according to the degree of elevation in titers (Table 1). Among neonates with a titer of ≥1:32, 43.3 percent (13/30) died. Among 10 neonates with a 4× elevation above the maternal level, 40 percent died compared to 16.6 percent of 12 whose titers were the same or less than their mother's. In the first group of six who died, five had these elevated titers to serotype 8 whereas in the group of five which survived only one had an elevated titer to serotype 8. In the six cases of death with paired sera, 66.7 percent of neonates and only 16.7 percent of the mothers had 4× elevated titers. In comparison among the 15 survivors, 33.3 percent of neonates and 40 percent of their mothers had elevated titers to at least one ureaplasma serotype. These groups are not appropriate for statistical analysis because of group size and the fact that an IgM specific titer of ≤1:16 may be indicative of infection in very premature neonates. In all cases where *U. urealyticum* infection was suspected, the neonates were treated with erythromycin and/or chloramphenicol.

Comparison of the Mean Antibody Titers

To assess whether there was a difference in the antibody titers observed in the neonates born with respiratory disease compared to normal neonates, the mean titers were calculated for each serotype (Table 2). In the RD case, the neonates had significantly elevated mean titers to serotypes 4, 7, and 8 when compared to the mean titers of the normal neonates and slight but not significant elevations to serotypes 3 and 6. Among the 21 pairs, ten neonates and seven mothers exhibited a 4× elevation with both observations occurring in three cases.

When the RD cases were assessed according to whether the infant survived or died (Table 2), certain differences in the mean titers were observed. The mean titer to serotype 3 was slightly elevated in all groups. With serotypes 4 and 8, the mean titers were significantly higher among neonates who died than among the survivors. For serotype 5, a significant elevation occurred only among the survivors. The elevation

TABLE 2
Mean Antibody Titers in Neonates and Mothers to *U. urealyticum* Serotypes

Serotype		Normal Pregnancy $N = 24$	Neonatal Respiratory Disease[a] All Patients $N = 54$ (I); $N = 21$ (M)	Survived $N = 32$ (I) $N = 15$ (M)	Died $N = 22$ (I); $N = 6$ (M)
1	I[b]	4.9	4.7	4.7	4.7
	M	4.8	5.1	5.5	4.1
2	I	6.0	7.5	7.2	8.1
	M	5.7	7.2	7.0	8.1
3	I	6.2	8.2	9.1	7.1
	M	6.2	8.8	9.2	8.1
4	I	4.5	6.5*	5.5	8.3*
	M	4.2	5.0	5.0	5.0
5	I	4.9	6.2	7.2*	5.0
	M	5.7	5.2	5.3	5.0
6	I	5.7	7.8	7.7	8.3
	M	5.8	8.5	7.3	12.7
7	I	5.5	9.2*	9.3	9.2
	M	5.2	6.1	6.1	6.4
8	I	4.9	9.3**	7.5	12.8*
	M	5.0	6.8	6.1	9.0

[a]Statistical significance $*p < .05$, $**p < .01$
[b]I = Infant; M = Mother

in mean titer to serotype 6 among the mothers of RD cases is strongly influenced by the high mean titer (12.7) among the six mothers whose neonates died (not statistically significant). For serotypes 6 and 7, the mean titer is elevated among the neonates to an equal degree in both groups.

Distribution of Antibody Titers in Neonates and Mothers

The distribution of the antibody titers, for serotypes in which a significant difference was observed, are shown for neonates and their mothers in Fig. 1. When the distribution is compared among the neonates for serotypes 4, 6, 7, and 8, there is a trend toward higher mean titers in neonates with respiratory disease. The degree of statistical significance is based on the comparison of the RD group, compared to the normal group. Sterotypes 4, 7, and 8 are the most significant in the RD group. When the distribution is compared for the mothers' titers, the mean titers to serotypes 6, 7, and 8 are elevated but not significantly.

Elevation in Antibody Titers by Gestational Age

Among the 54 RD neonates, titers of $\geq$1:32 to *U. urealyticum* serotypes were evaluated according to gestational age. In the group of 14 neonates born between 22–27 weeks of gestation, 64.3 percent had a titer of 1:32 to at least one serotype. Among 25 neonates of 28–35 weeks' gestation, 36.0 percent were elevated and among 15 of those born after 36 weeks' gestation, 80.0 percent had elevated titers. The incidence of titers of $\geq$ 1:32 were all significantly higher than the 4.2 percent observed in normal neonates.

In the cases of the very premature neonates, 22–30 weeks' gestation, the elevation in titers tended to be low, e.g., 1:8 to 16 or 1:8 to 1:32 for mother vs. neonate, but with some neonates as high as 1:256. In the infants born after 30 weeks, the titers ranged from 1:8 to 1:32 up to 1:8 to 1:128. Since the mothers can have high titers due to IgG which crosses the placenta, a high neonatal titer may not be indicative of neonatal infection.

DISCUSSION

U. urealyticum has been considered an infectious agent in pregnancy, leading to a poor outcome [1–11], but a causal role is difficult to assess. Colonization may occur before or after the death of the fetus, after an extended period of ruptured membranes, or the organism may be aspirated during delivery. Second, the inflammatory response in lungs and placenta may be due to other microorganisms not detected. However, elevation in IgM antibodies to the same ureaplasma serotype isolated from the lung [11] is indicative of true infection. In the bovine model, inoculation of pregnant cows with *U. diversum* at five months of gestation resulted in abortion or severe respiratory distress. The organism was isolated and an inflammatory response was observed in the lungs, similar to that observed in humans [12]. These observations support the hypothesis that the living fetus may be infected with ureaplasmas *in utero.*

In this study elevated antibody responses to specific *U. urealyticum* serotypes were found in infants at delivery. A number of situations are therefore possible. The mother had the infection prior to conception with circulating IgG antibody which crossed to the fetus, resulting in equal titers. In the second group, infection is established in the mother prior to conception or during pregnancy such that she has or develops an antibody response of the IgM class. Because these antibodies do not cross to the fetus, the mother has a higher titer than the neonate. In the third case, infection occurs on the fetal side on the immunological barrier. The fetus develops its own IgM specific antibody response which does not cross to the maternal circulation. The neonate, therefore, has an elevated titer over the maternal level. In a few cases this elevated titer was proven to be IgM-specific [11; unpublished results]. Finally, the mother could have IgG plus IgM. The fetus acquires maternal IgG but synthesizes its own IgM, which results in high titers in both mother and fetus. The situation is complex because of the large number of serotypes which could elicit these antibody responses. In one case, the mother had elevated titers to serotypes 6 and 7, while the neonate had elevated titers to serotypes 2 and 4.

The mean titers were assessed to determine which serotypes elicited a significant antibody response. A small elevation in the mean titer was observed to serotype 3 in both the normal and RD groups and was also found in the spontaneous abortion group. Since 80 percent of the local population, whether fertile or infertile, carry serotype 3 compared to 20 percent or less for the other serotypes [16], one would expect an antibody response comparable in all groups. In the RD cases, the neonates had elevated titers to serotypes 4, 7, and 8, but the mothers did not to a significant degree. In mycoplasma-positive women with a history of pregnancy loss (PL), the maternal titers were elevated to serotypes 4 and 8, and to 6 and 8 in the neonates [15]. Since the significantly elevated antibody responses do not occur to serotype 3 but to serotypes 4, 6, 7, and 8, the data suggest that serotype 3 may exist as a commensal while serotypes 4, 6, and particularly 8 may be more pathogenic.

In abortion, *U. urealyticum* appears to play a causal role. An inflammatory response in the lungs or lesion in the tracheal epithelium may or may not be observed

[8]. In our studies, neonates of 22–28 weeks' gestation exhibited fourfold higher titers than their mothers. This fact supports the concept that the fetus is infected *in utero,* eliciting its own immune response. In our studies of abortion associated with genital mycoplasmas there is a marked trend in which 40 percent of pregnancies are lost later in the second trimester. Why some infected fetuses are live rather than aborted or stillborn is not known. However, it is possible that those infants in whom antibodies develop or who acquire antibodies from their mothers, cope with the infection better, resulting in a live birth. Finally, among the healthy neonates of erythromycin-treated PL mothers [17], the antibody response to serotype 8 was most significant as it was for RD neonates who died. This fact suggests that erythromycin therapy during gestation prevented severe disease in the fetus, resulting in healthy neonates.

In conclusion, the development of an immune response to the presence of *U. urealyticum* supports the concept that this organism is capable of causing fetal/neonatal infection. Women may be colonized with *U. urealyticum* but exhibit no clinical signs of disease. However, in certain women and during pregnancy, the organism may cause infection in the mother and/or the fetus which results in abortion, stillbirth, prematurity, and severe congenital pneumonia or chronic respiratory disease in the neonate.

ACKNOWLEDGEMENTS

The authors wish to acknowledge the support and cooperation of the doctors and staff in the Department of Neonatology and the staff in the Blood Bank, The Hospital for Sick Children.

This research was supported by Ontario Provincial Health Grant PR-547 and the Research Institute, The Hospital for Sick Children.

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