Broadly Reactive Immunofluorescence Test for Measurement of Immunoglobulin M and G Antibodies to *Ureaplasma urealyticum* in Infant and Adult Sera

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The indirect immunofluorescence test was used to measure immunoglobulin M (IgM) and IgG antibodies to acetone-fixed *Ureaplasma urealyticum* organisms in sera from 128 adults with genital infections and from 713 symptomatic newborns and babies 1 day to 18 months old. Thirty-four percent of the adults had demonstrable IgG antibody to ureaplasma. IgM antibody was detected in 2 of the adult sera and in 17 of the infant sera. These babies were divided into two distinct groups. Ten of the infants presented at birth with various physical findings, whereas the onset of symptoms for the other 7 occurred 3 to 13 weeks after birth, and the major clinical finding in 6 of the 7 was respiratory distress. The results of this study suggested that *U. urealyticum* infection may be associated with fetal damage and infant pneumonia, and if this is substantiated, the indirect immunofluorescence test employing acetone-fixed antigen to measure IgM antibody to *U. urealyticum* may be an important diagnostic tool.

Ureaplasma urealyticum has been shown to be a cause of urethritis in males (19), and it is a common contaminant of the female genital tract. Isolation of this agent has been associated with spontaneous abortion, inflammation of the placenta, intrauterine fetal growth retardation, early neonatal death, and infant pneumonia (4, 10, 17, 20). However, attempts to implicate *U. urealyticum* as a venereal disease agent capable of causing in utero infection and infant morbidity and mortality have been hampered by the fact that infants and adults without apparent symptoms can also be colonized with these organisms (6, 20). At least 14 types of *U. urealyticum* have been described (18), and it has been suggested the perhaps some types cause morbidity whereas others are part of the normal flora (20). Demonstration of fourfold antibody titer rises between acute- and convalescent-phase serum specimens or detection of immunoglobulin M (IgM) antibody to *U. urealyticum* would be most helpful in firmly establishing this agent as the cause of infection. However, most serological methods developed thus far are relatively type specific (2, 13-15), which limits their usefulness as diagnostic tools. Stemke and Robertson (18) have recently described an indirect immunofluorescence (IIF) test employing a Triton X-100 treated antigen, which appears to be group specific. In the present study, we used acetone-fixed *U. urealyticum* organisms in an IIF test to

measure antibody responses in sera from adults with genital infections and from symptomatic infants.

MATERIALS AND METHODS

Sera examined. Included in the study were 85 sera submitted for syphilis serology from males attending a venereal disease clinic, 43 sera from males with urethritis who were culture negative for *Neisseria gonorrhoeae* and *Chlamydia trachomatis*, and 713 sera from newborns and infants from 1 day to 18 months old with suspected viral or chlamydial infection, most of whom had been screened previously for the presence of IgM antibody to *Toxoplasma gondii*, rubella virus, cytomegalovirus, herpes simplex virus, and chlamydiae (7). Fourteen serum pairs from patients with *Mycoplasma pneumoniae* infection were used as one set of specificity controls.

Rabbit antisera were prepared to whole organisms of three types of *U. urealyticum*: types 3, 4, and 8. The immunogens were cultivated in dialysate broth supplemented with 30 mM urea, 100 mM 2-(N-morpholino)ethane sulfonic acid at pH 6.3, 2% "agamma" rabbit serum, and 1 mM sodium sulfite (9). Immunization was carried out as described previously (8), using 1 to 3 mg of ureaplasmic protein per rabbit.

Two polyclonal monospecific rabbit antisera to individual common antigens of *U. urealyticum* were used. One antiserum was to the urease enzyme (electrophoretic mobility relative to bovine albumin, 0.53) and the other to a cytoplasmic component of 0.43 mobility. These antisera were prepared by using precipitin lines excised from crossed-immunoelectrophoresis slides as immunogens (1). Each of the antisera was monospecific

ic only to its homologous antigen. The antigenic determinants were common to the species since the antisera gave a single precipitin line with antigens prepared from the eight serotypes.

Two additional monospecific antisera were used as specificity controls: one prepared to a cell wall antigen of *Actinomyces israelii* (G. Ayakawa and G. Kenny, submitted for publication) and the other to an antigen in *Trypanosoma brucei* var. *brucei* (G. E. Kenny, unpublished data). These two antisera were used to provide controls for the possibility of mycobacterial antibodies cross-reacting with *U. urealyticum* because the monospecific antiserum immunization protocol employed Freund complete adjuvant. Antisera to *M. pneumoniae*, *Mycoplasma neurolyticum*, and calf serum were prepared as described elsewhere (8).

***U. urealyticum* antigen preparation.** One 7-day-old bottle culture (8 oz [240 ml]) of uninfected McCoy cells was trypsinized, and the cells were collected in 10 ml of Eagle minimal essential medium in Earle balanced salt solution containing 10% fetal bovine serum (FBS). Sixty 8-spot slides (Bellco Glass, Inc., Vineland, N.J.) were inoculated with 0.02 ml of cell suspension per spot, and the slides were placed in a moist chamber, incubated at 35°C (5% CO₂, 80% humidity) for 24 h, air dried, fixed in acetone for 10 min at room temperature, and stored at -60°C. Before being inoculated with *U. urealyticum*, the slides were brought to room temperature, rinsed in distilled water to remove the salts, and air dried.

***U. urealyticum* type 4 (ATCC 27816)** was propagated in Shepard's standard fluid medium 10-B (16) containing 10% FBS instead of horse serum. Before use, the medium (stored at -20°C) was warmed to 35°C for 30 min, which resulted in some precipitation of the yeast extract. This precipitate was then removed by centrifugation at $2,800 \times g$ for 2 h at 35°C. (Failure to remove this precipitate resulted in nonspecific reactivity with some sera in the IIF test, which masked specific staining of the antigen.) A 2-oz (60 ml) prescription bottle containing 10 ml of medium was inoculated with 3 ml of *U. urealyticum* stock previously stored at -60°C in 10% dimethyl sulfoxide. When the pH had risen to 7.2, as indicated by the phenol red indicator (1 to 2 days), the 10 ml was transferred to an 8-oz (240-ml) bottle containing 40 ml of fresh medium. Color change was observed in 24 h, and the 40 ml was inoculated into a 32-oz (960-ml) bottle containing 160 ml of medium. The pH rose in approximately 8 h, and at 24 h the medium was centrifuged at $2,800 \times g$ for 2 h at 4°C, and all but approximately 0.1 ml of the supernatant fluid was removed. The sediment was reconstituted in 10 ml of phosphate-buffered saline containing 2% FBS, and 0.02 ml was inoculated onto each well of the slides previously seeded with McCoy cells. The slides were air dried, fixed in acetone for 10 min at room temperature, and stored at -60°C.

***M. pneumoniae* antigen preparation.** One 16-oz (480-ml) bottle containing 20 ml of PPLO broth, 25% yeast extract, 20% FBS, and phenol red indicator was inoculated with 0.1 ml of *M. pneumoniae* (AP 164 strain) stock. The bottle was incubated, flat side down, at 35°C until a yellow color change in the medium occurred (5 days). At this time, an almost confluent sheet of colonies was visible on the glass surface at $\times 100$ magnification. The medium was removed, and

the colonies were scraped into 0.8 ml of the reserved fluids and pipetted vigorously to break up clumps. One 7-day-old bottle culture (240 ml) of uninfected McCoy cells was trypsinized, and the cells were reconstituted in 1 ml of cell culture growth medium and added to the concentrated *M. pneumoniae* fluids. One drop (ca. 0.005 ml) of the suspension was added to each well of 8-spot slides, and the slides were incubated on moist trays in a CO₂ incubator for 24 h. The slides were then air dried, fixed in room-temperature acetone for 10 min, and stored at -60°C. Efforts to propagate ureaplasma colonies on slides as a source of antigen, as was done for *M. pneumoniae*, were not successful.

Immunofluorescence procedures. The IIF methods for determination of IgG and IgM antibodies have been described previously (5, 7). All sera reacting in the IgM test were absorbed with aggregated human IgG (3) and retested for presence of IgM antibody. The adult sera were first screened at a 1:8 dilution for IgG antibody to *U. urealyticum*, and the positive sera were then tested for IgM antibody. The infant sera were screened in the opposite manner. The reactions were read on a Zeiss fluorescence microscope at $\times 250$ magnification, utilizing incident light.

RESULTS

The McCoy cells in the slide preparations served as nonspecific controls for *U. urealyticum* and *M. pneumoniae* staining reactions. Individual organisms fluoresced with positive sera in the ureaplasma test (Fig. 1A) and, in most instances, were not visible with negative sera. Staining of various-sized colonies of *M. pneumoniae*, which had developed during the overnight incubation of the organisms on the slides, was observed with sera possessing antibody to this agent (Fig. 1B).

Of 85 sera submitted for syphilis serology, 32 (38%) possessed IgG antibody to *U. urealyticum*, and IgM antibody was demonstrable in 2 of these. IgG antibody to *U. urealyticum* was present in 13 of 43 sera (30%) from males with nongonococcal and nonchlamydial urethritis, but no IgM antibody was detected. Of 713 infant sera tested, 17 (2.4%) possessed IgM antibody to *U. urealyticum*. The clinical findings in the 10 babies with onsets of symptoms at birth varied, whereas evidence of respiratory infection was the major finding in 6 of 7 infants with onsets 3 to 13 weeks after birth. IgM antibody to rubella virus had previously been detected in serum from infant no. 11, and chlamydial IgM antibody had been demonstrated in sera from patients no. 15 and no. 16 (Table 1).

Twenty-two sera from adults with antibody to *U. urealyticum*, including the two with IgM antibody, were tested for presence of antibody to *M. pneumoniae*. IgG antibody to this agent was demonstrated in 15 sera (68%), and 8 of these (53%) were also positive in the IgM test for *M. pneumoniae*. The 14 serum pairs from pa-

FIG. 1. (A) Positive immunofluorescence staining for *U. urealyticum* IgM antibody ($\times 625$). (B) Positive immunofluorescence staining for *M. pneumoniae* IgM antibody ($\times 625$).

tients with respiratory disease in whom fourfold antibody titer rises to *M. pneumoniae* were demonstrated by complement fixation were tested for *U. urealyticum* antibody, and 4 pairs

(29%) showed stationary IgG titers, whereas no IgM antibody was detected.

Rabbit antisera to *U. urealyticum* and other antigens reacted as shown in Table 2. The fluorescent-antibody technique showed no type specificity since similar titers were obtained with all antisera against whole organisms. More importantly, the test detected antibodies to both urease and the other cytoplasmic components, indicating that the fixation had revealed intracellular antigens. Monospecific antisera to irrelevant antigens did not react, indicating that mycobacterial antibodies did not cause significant cross-reactions. Thus, we conclude that the test as described could easily measure antibody to urease, but we cannot exclude the possibility that several other antibodies could be detected as well. The test was specific in that no reactions were obtained with rabbit antisera to *M. pneumoniae* and *M. neurolyticum* (both antisera produced 6 to 10 precipitin lines against their homologous antigens), nor did rabbit antiserum to calf serum react even though FBS was used for propagation of *U. urealyticum* and for suspension of the organisms.

DISCUSSION

In this study, IgG antibody to *U. urealyticum* was demonstrable in 34% of patients with genital infections and in 29% of those with *M. pneumoniae* infections, which indicates that a significant proportion of the adult population has had infection at some time with *U. urealyticum*. However, the usefulness of the IgM antibody test in diagnosing adult ureaplasma infection was difficult to assess. Only two patients with genital infections had demonstrable IgM antibody which would be expected to indicate recent or current infection with this agent. It is not known, of course, how many of these patients were actually infected with *U. urealyticum* at the time of specimen collection. Acute- and convalescent-phase serum pairs were not available from this group, and it is possible that rises in IgG antibody to *U. urealyticum* could have been demonstrated in some cases. It is more probable, however, that, as is the case with other sexually transmitted infections, neither IgM nor IgG antibody to ureaplasmas is stimulated with any regularity in recurrent infection.

Seventeen of the infant sera possessed IgM antibody to *U. urealyticum*. Findings in the newborns were varied and included severe neurological disorders. However, clinical findings in patients with onset of symptoms occurring 3 to 13 weeks after birth were limited to respiratory infection, with the exception of patient no. 13, suggesting that infection with *U. urealyticum* in utero may cause damage to various organs but that if primary exposure to this agent occurs in

TABLE 1. Infants with IgM antibody to *U. urealyticum*

Patient no. ^a	Age at onset	Age at specimen collection	Clinical findings ^b	IIF titer	
				IgM	IgG
1	Birth	1 day	Intrapartum asphyxia	8	<8
2	Birth	1 day	SGA, sepsis, thrombocytopenia	128	128
3	Birth	3 days	SGA, severe hypoglycemia	8	8
4	Birth	5 days	Failure to thrive	8	<8
5	Birth	5 days	Petechiae, elevated total IgM	8	<8
6	Birth	7 days	Jaundice, failure to thrive	32	8
7	Birth	8 days	High bilirubin, seizures	8	32
8	Birth	10 days	Small placenta, seizures, apnea	128	8
9	Birth	12 days	Intrauterine asphyxia, seizures, cerebral edema	128	32
10	Birth	3 wks	Seizures, abnormal EEG	≥512	128
11	3 wks	5 wks	Pneumonia	8	8
12	4 wks	4 wks	Conjunctivitis, lung infiltrate	128	8
13	4 wks	5 wks	Seizures, coma	8	<8
14	5 wks	6 wks	Pneumonia	32	8
15	5 wks	8 wks	URI 3 wks, now has upper lobe pneumonia	8	8
16	Not available	8 wks	Conjunctivitis, pneumonia	32	128
17	13 wks	16 wks	Conjunctivitis, cough	32	<8

^a Rubella virus IgM antibody was detected in the serum from patient no. 11. *C. trachomatis* IgM antibody was present in serum specimens from patients no. 15 and no. 16. *C. trachomatis* infection was suspected in patients no. 11, 12, 15, 16, and 17.

^b SGA, Small for gestational age; URI, upper respiratory infection; EEG, electroencephalogram.

the birth canal, infection of mucous membranes may result. Five of the six infants with respiratory disease who possessed IgM antibody to *U.*

urealyticum had been diagnosed as having chlamydial pneumonia, and chlamydial IgM antibody had previously been detected in two of these patients, suggesting a dual infection. A nonspecific cross-reaction to ureaplasmas stimulated by chlamydial infection does not appear likely, since seven of the sera in this study possessed IgM antibody to *C. trachomatis* but not to *U. urealyticum*. Stagno et al. (17) found that it was impossible to clinically distinguish infant pneumonitis due to *C. trachomatis* from that due to *U. urealyticum*. Both agents were isolated from 2 of 104 patients in their study.

IgG antibody was detected in all five of the immune rabbit sera made to different ureaplasma antigens and in 32% of 142 adult sera, which would imply that this antigen is broadly reactive for ureaplasma antibody. Previously described serological methods have usually utilized untreated organisms, with resulting type specificity. Our antigen was fixed in acetone, which presumably exposed group-specific components within the organism.

Sera from patients with pancreatitis have been shown to react non-specifically with the lipid antigen of *M. pneumoniae*, and this antibody response is mainly of the IgM class (11, 12). In this study, none of the 14 pairs of sera from patients with *M. pneumoniae* infection who possessed IgG antibody to *U. urealyticum* reacted in the IgM test for *U. urealyticum* antibody. In contrast, of 15 genitally infected patients with both *M. pneumoniae* and *U. urealyticum* IgG antibodies, 8 also had demonstrable IgM antibody to *M. pneumoniae*. Thus, although the

TABLE 2. IIF results with sera from rabbits inoculated with *U. urealyticum* and other antigens

Animal no.	Immunizing antigen	IIF titer
1	Preimmunization	<8
	<i>U. urealyticum</i> type 3 whole cells	≥512
2	Preimmunization	<8
	<i>U. urealyticum</i> type 4 whole cells	≥512
3	Preimmunization	<8
	<i>U. urealyticum</i> type 8 whole cells	≥512
4	Preimmunization	<8
	Precipitin peak for a cytoplasmic antigen of <i>U. urealyticum</i>	128
5	Preimmunization	<8
	Urease precipitin peak	128
6	<i>A. israelii</i> cell wall antigen	<8
7	<i>T. brucei</i> var. <i>brucei</i> antigen	±8 ^a
8	<i>M. pneumoniae</i>	<8
9	<i>M. neurolyticum</i>	<8
10	Calf serum	<8

^a Minimal reactivity identical to that seen with uninfected McCoy cells.

ureaplasma IgM test appeared to be specific for ureaplasmas, this was not the case with IgM reactions for *M. pneumoniae*. However, IgM responses to *M. pneumoniae* have been shown to be quite long lasting, and the antibody detected may have resulted from previous infection. It is also possible that the IgM response is a polyclonal activation response to previous exposure to *M. pneumoniae* antigens.

In general, isolation of the causative agent in ill newborns and infants has been shown to be a more sensitive technique than detection of IgM antibodies, but isolation of *U. urealyticum* does not necessarily implicate this organism as the cause of the disease, since this agent has also been recovered from asymptomatic infants. Our results suggest that *U. urealyticum* infection may be a significant cause of newborn morbidity and infant pneumonia and that the IIF-IgM antibody test with an acetone-fixed antigen could be an important diagnostic tool. This will be additionally explored by studies which correlate clinical findings and isolation rates with antibody detection.

ACKNOWLEDGMENTS

This study was supported in part by Public Health Service grants AI-15990 and AI-06720 from the National Institute of Allergy and Infectious Diseases.

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