Isolation and Characterization of *Mycoplasma genitalium* Strains from the Human Respiratory Tract

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Mycoplasma genitalium, an organism first isolated from the urethras of two men with nongonococcal urethritis, has been found in throat specimens from military recruits participating in an inactivated *Mycoplasma pneumoniae* vaccine field trial in 1974–1975. Four of 16 preserved throat isolates, previously identified as strains of *M. pneumoniae*, have now been shown to be mixtures of *M. pneumoniae* and *M. genitalium*. Purification of these mixed mycoplasmas by selection of single colonies confirmed the presence of *M. genitalium*. Identification of *M. genitalium* was based upon the occurrence of a species-specific 140-kilodalton protein adhesin in these isolates and their serologic reactivity to an *M. genitalium* antiserum. The frequent occurrence of both *M. pneumoniae* and *M. genitalium* in a number of these throat specimens, in combination with their shared antigenic cross-reactivities, suggests the likelihood that *M. genitalium* strains are easily missed in the usual laboratory identification procedures. What role *M. genitalium* may play in human respiratory disease remains to be determined.

A new mycoplasma, distinct from all other known mycoplasmas, was isolated in 1980 from urethral specimens of two men with nongonococcal urethritis (NGU) (31). The basic morphologic, serologic, and genetic features of this organism were later defined, and because of an apparent relationship between its host tissue location and potential to cause disease, it was named *Mycoplasma genitalium* (32). This mycoplasma shared numerous properties, such as the flask shape and terminal tiplike organelle, with strains of *Mycoplasma pneumoniae* (1, 10, 12), the etiologic agent of primary atypical pneumonia of humans. Both microorganisms exhibited adherence capabilities in that they attached to various types of tissue cells, erythrocytes, and inert surfaces (glass and plastic). In addition, *M. genitalium* and *M. pneumoniae* strains shared cross-reactions in a variety of serologic tests (2–4, 6, 16, 18, 27). More recently, *M. genitalium* strains were found to possess a species-specific 140-kilodalton (kDa) protein that is strongly immunogenic and apparently functions in a manner similar to the P1 protein (170 kDa) that is the major cytoadhesin of *M. pneumoniae* (7, 13, 15, 22, 24). Interestingly, these two proteins (140 and 170 kDa) also share cross-reactive epitopes (6, 23).

Although *M. genitalium* strains were first isolated from the human urogenital tract, no other definite isolations have been reported since the 1981 publication (31). Likewise, despite experimental findings that the organism could induce genital tract infections in a variety of nonhuman primates (20, 26, 29, 33), direct evidence linking this mycoplasma to NGU in humans is lacking.

In 1974–1975, a field trial of a Formalin-inactivated *M. pneumoniae* vaccine was carried out in United States military recruits (34). Mycoplasmas were isolated from frozen throat specimens of a number of the recruits hospitalized with pneumonia after completion of the field trial (30), and these mycoplasmas were preserved by lyophilization at the National Institutes of Health. This report describes recently obtained evidence that some of these throat isolates contain

M. genitalium and retrospectively identifies the human oral cavity as an additional, and perhaps predominant, locale for the organism.

MATERIALS AND METHODS

Patients and specimen sampling. A large group (7,861) of recruits at the Marine Corps depot at Parris Island, South Carolina, entered into a vaccine trial in 1974 with a Formalin-inactivated *M. pneumoniae* strain (CL-8) (34). Throat swabs from individuals receiving vaccine or a placebo, and from all participants hospitalized with pneumonia, were placed in a transport medium and frozen in dry ice. Within 1 or 2 days after collection, the throat specimens were thawed rapidly and tested in conventional culture medium for the presence of *M. pneumoniae* (34). The specimens were then refrozen at -70°C . Shortly after completion of the field trial, a large number of the throat specimens (but predominantly those that gave negative culture attempts for *M. pneumoniae*) were shipped in the frozen state to the National Institutes of Health. In 1977, 600 or more of these specimens were inoculated into a newly developed culture medium (SP-4), and approximately 30 to 40% of those specimens negative for *M. pneumoniae* in the initial cultivation attempts yielded *M. pneumoniae* isolates (30). These strains, which were identified at the time as *M. pneumoniae* by immunofluorescence tests on agar plates (8), were either preserved by lyophilization or frozen at -70°C .

Immunological screening. Lyophils of 16 clinical isolates previously identified as *M. pneumoniae* from the earlier serologic tests were sent to the University of Texas Health Science Center, San Antonio, in late 1987. Fifteen of the 16 strains had been recovered from patients hospitalized with pneumonia in the 1974–1975 field trial. Limited purification of the mycoplasma strains was carried out as follows. Each strain was grown in SP-4 broth for 7 to 14 days at 37°C , the broth cultures were subsequently subjected to serial 10-fold dilution in fresh culture medium, and individual samples of each dilution were plated onto a standard Edward-type mycoplasma agar medium (9). After the appearance of

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mycoplasma colonies on the agar plates, single, well-isolated individual colonies were transferred to fresh SP-4 broth medium, and stock strains were prepared. For immunological screening, stock strains of the 16 isolates were grown at 37°C in 32-oz. (about 950-ml) bottles containing 70 ml of SP-4 medium. Mycoplasmas were washed and harvested by centrifugation for 20 min at $9,500 \times g$. Cell pellets were solubilized in buffer containing sodium dodecyl sulfate and β -mercaptoethanol (23) prior to analysis by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Mycoplasma proteins were electrophoretically transferred to nitrocellulose membranes and examined for immunoreactivity using monoclonal antibodies generated against the 170-kDa P1 protein of *M. pneumoniae* and the 140-kDa protein of *M. genitalium*. Experimental conditions and monoclonal antibody classes were selected to prevent cross-reactive immunological patterns between these *Mycoplasma* species (22, 23).

Serologic testing. Disk growth inhibition (GI) tests (5) and metabolism inhibition (MI) tests (28) were performed in Frederick, Md., on the five original unpurified clinical isolates (TW10-5, TW10-6, TW48-5, R32, and TW8-6) previously identified as *M. pneumoniae* and on each of the four isolates purified by single-colony selection in San Antonio. The MI and GI test procedures utilized National Institutes of Health reference antiserum prepared in mules to the FH strain of *M. pneumoniae*, or antiserum prepared in rabbits to the G-37 strain of *M. genitalium*. Control MI and GI tests were also carried out with the respective FH and G-37 type strains of the two *Mycoplasma* species.

RESULTS

Uncloned *M. pneumoniae* isolates derived from throat specimens of military recruits were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting to monitor possible variations in protein and antigenic profiles. Examination of the Coomassie brilliant blue-stained polypeptides of these strains revealed unexpected differences in the 170-kDa region considered to represent the essential cytoadhesin protein P1 of virulent *M. pneumoniae*.

In Fig. 1 (I), lanes A, B, and C display the 170-kDa P1 protein and other polypeptides which are typical of *M. pneumoniae* profiles. The absence of the P1 protein in lanes D (R32 isolate) and E (*M. genitalium* G-37) coincides with the appearance of a polypeptide of 140 kDa. Note that lane C (unpurified TW10-5 isolate) contains both the 170- and 140-kDa proteins. Several other protein differences can be detected as previously reported (2, 22, 23).

Since *M. genitalium* has been implicated in human disease and possesses a major antigen of 140 kDa, we performed immunoblots using specific monoclonal antibodies directed against distinct epitopes of the 170- and 140-kDa proteins (23). Figure 1 (II) clearly demonstrates the presence of the P1 protein of *M. pneumoniae* in the control *M. pneumoniae* strain and in the TW8-6 and uncloned TW10-5 isolates (lanes A, B, and C₁), while the three other lanes (C₂ to E, containing uncloned TW10-5, R32, and the *M. genitalium* G-37 strain, respectively) reveal the 140-kDa protein of *M. genitalium*.

Serologic comparisons of five of the throat isolates, both before and after purification by single-colony selection, were performed by MI and GI tests. The results (Table 1) confirmed the polyacrylamide gel electrophoresis and immunoblot findings. The uncloned mixed TW10-5 isolate showed

FIG. 1. (I) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis protein profile of clinical isolates from throat cultures stained with Coomassie brilliant blue. The left lane contains molecular weight standards. Lanes: A, *M. pneumoniae* M129-B16; B, isolate TW8-6; C, isolate TW10-5 uncloned; D, isolate R32; E, *M. genitalium* G-37. (II) Immunoblot of a similar gel reacted with monoclonal antibody to the cytoadhesin P1 protein of *M. pneumoniae* (lanes A through C₁) and to the 140-kDa protein of *M. genitalium* (lanes C₂ through E).

considerable cross-reactions in the MI test to both *M. pneumoniae* and *M. genitalium* antisera. In GI tests no clear zones of inhibition were observed on agar plates because the *M. pneumoniae* component of the mixture grew in the presence of *M. genitalium* antiserum and the reverse reaction occurred in the presence of *M. pneumoniae*-specific antiserum. Similar responses (not shown) occurred with unpurified strains of the TW10-6, TW48-5, and R32 isolates. When purified strains were tested in each serologic procedure, the results indicated that the *M. genitalium* component of the mixture seen in the TW10-5, TW10-6, and R32 isolates had been selected out (Table 1) (TW48-5 data not shown). The resulting strains reacted to high titer against *M. genitalium*-specific antiserum in MI tests and exhibited only minor cross-reactions (titers less than 1:80) in the presence of *M. pneumoniae* antiserum. GI test results were similar to findings observed with the control *M. genitalium* organism. The purified TW8-6 isolate was also subjected to a similar serologic analysis, and the results showed this to be a strain of *M. pneumoniae* (Table 1). The other 11 isolates in the group were characterized serologically as *M. pneumoniae*.

DISCUSSION

The discovery and isolation of *M. genitalium* from the urethras of patients with NGU in 1980 were thought at the time to offer support to the idea that other tetracycline-susceptible organisms, in addition to *Chlamydia trachomatis* and *Ureaplasma urealyticum*, might account for as much as 25% of unexplained NGU infections. The pathogenicity of the two *M. genitalium* strains for the urogenital tract of both male and female hosts was amply confirmed in numerous experimental studies performed on a variety of nonhuman primates (20, 26, 29, 33). In addition, experimental infections in chimpanzees have been correlated with pathophysiology, with specific serum antibody conversion, and, more recently, with a strong rise in serum antibody titer during acute-convalescent stages to the specific 140-kDa protein

TABLE 1. Detection of *M. pneumoniae* and *M. genitalium* strains from human throat specimens by two serologic procedures

Strains used as antigens	Serologic results of antigens tested against specific antisera			
	MI titer ^a		GI zone ^b (mm)	
	<i>M. pneumoniae</i> FH	<i>M. genitalium</i> G-37	<i>M. pneumoniae</i> FH	<i>M. genitalium</i> G-37
Controls				
<i>M. pneumoniae</i> FH	10,240	80	6	0
<i>M. genitalium</i> G37	80	1,280	0	6
Throat specimens				
TW10-5 uncloned	10,240	640	0	0
TW10-5 SCS ^c	<80	2,560	0	6
TW10-6 SCS	<80	1,280	0	5
R32 SCS	<80	1,280	0	6
TW8-6 SCS	5,120	320	6	0

^a Results of MI test performed in microtiter plates containing constant numbers of broth-grown organisms and dilutions of specific antiserum in culture medium containing a pH indicator. Values given are the reciprocal titers of the antiserum dilution showing metabolism inhibition.

^b Results of GI procedure performed with mycoplasmas grown on agar plates. Values given represent zones of growth inhibition (in millimeters) around paper disks saturated with specific antiserum.

^c SCS, Single-colony selection of mycoplasmas from strains grown on agar medium.

which is thought to function as an attachment moiety for the organism (13, 22, 23).

Despite the occurrence of the organism in patients with urethritis and the ability of *M. genitalium* to induce experimental infections in both male and female hosts, difficulties in direct isolation of the organism from urogenital specimens in a variety of clinical settings have raised concerns as to whether the organism occurs in these sites or whether such difficulties involve deficiencies in culture media or other factors in the laboratory. Current support for the role of the organism in human disease has been based primarily on serologic associations (17, 19, 21, 25). However, in view of the known cross-reactions between *M. genitalium* and *M. pneumoniae* and the undefined nature of the antigens usually employed in these serologic tests, these associations must be viewed with caution.

Difficulties in the cultivation of *M. genitalium* from clinical specimens and problems in interpretation of serologic findings in these circumstances have prompted considerable interest in the use of DNA probes to detect the organism in clinical specimens. Although common DNA sequences have been found in the genomes of *M. genitalium* and *M. pneumoniae* (35), specific nick-translated whole genomic probes from both organisms were shown in control dot-blot hybridization procedures with known mycoplasmas to be specific for either *M. pneumoniae* or *M. genitalium* (14). The first use of an *M. genitalium* DNA probe on clinical specimens obtained from men attending a sexually transmitted disease clinic was recently reported (11). In this study, occurrence of *M. genitalium* varied from a low of 10% in individuals with chlamydia-positive NGU to 27% in those with persistent or recurrent NGU. Twelve percent of those participants without urethritis were positive with the probe. With the DNA probe, *M. genitalium* appeared to be much more frequent in homosexual men (30%) than in heterosexual men (11%), leading the authors to suggest that the organism may reside in the gastrointestinal tract (11).

The occurrence and identification of *M. genitalium* in human throat specimens reported here would appear to provide some explanation for difficulties in primary isolation of the organism from the urogenital tract. Obviously, these findings will necessitate further confirmation of *M. genitalium* in recently obtained throat specimens. Our observation that *M. genitalium* can be directly isolated from the human

respiratory tract, frequently in close association with *M. pneumoniae*, also suggests other important ramifications to the biology and laboratory diagnosis of each of the two organisms. The biological and immunological similarities which *M. genitalium* and *M. pneumoniae* share certainly have complicated earlier isolations and diagnoses. It is obvious that suspected *M. pneumoniae* or *M. genitalium* isolates from the respiratory tract cannot be definitively identified in any of the conventional laboratory techniques without some type of cloning or purification procedure. The coexistence of *M. genitalium* and *M. pneumoniae* dramatizes the need to carefully reanalyze the epidemiology of mycoplasma-mediated primary atypical pneumonia in humans, since it is possible that *M. genitalium* strains may act in a synergistic role similar to other types of mixed infections, or the organism itself may be capable of inducing respiratory and other mycoplasma-mediated diseases. In this regard, we have documented recent evidence (unpublished data) suggesting the occurrence of *M. genitalium* in synovial fluids of a patient with arthritis. Thus, the isolation of *M. genitalium* from throat specimens expands information on the localized tissue tropism of the organism and on its potential to produce disease at these sites.

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