Characterization of Spiroplasma mirum (Suckling Mouse Cataract Agent) in a Rabbit Lens Cell Culture

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Spiroplasma mirum (suckling mouse cataract agent) was studied in an epithelial cell line AG-4676, derived from rabbit eye lens. Rabbit eye lens is a natural target tissue of S. mirum infection. The organism grew rapidly in this cell line, reaching titers of 10^7 to 10^9 color change units per ml at 7 days after infection. This is the same level as that achieved in SP-4 medium designed specifically for S. mirum. No lag period was apparent in growth in AG-4676. S. mirum did not grow in Dulbecco minimal essential medium–10% fetal bovine serum, the medium for AG-4676, indicating the need for cells or a cellular product. S. mirum-infected AG-4676 cells exhibited vacuolization and granulation and an increase in polynucleation compared with uninfected controls (36/100 versus 14/100, P < 0.001). Infection significantly decreased the growth rate of AG-4676, especially late in the growth cycle. In a representative experiment, growth of AG-4676 at 11 days was reduced from 9 \times 10^8 to 2 \times 10^8 cells by S. mirum infection. S. mirum grew to high titers in conditioned medium of AG-4676, obtained from cell-free supernatants of 1- to 5-day-old AG-4676 cultures. This growth promotion was not due to osmotic conditioning of the medium. Preliminary characterization of this growth promotion substance showed it to be active after 0.22-μm filtration, heating at 56°C for 30 min, freezing and thawing, and dilution at 10^{-1} but not 10^{-2}. AG-4676-propagated S. mirum produced death or cataracts in suckling Wistar rats at the same frequency (55/60, 91.7%) as SP-4-propagated organisms (60/65, 92.3%).

Spiroplasma mirum, formerly known as suckling mouse cataract agent (SMCA) was originally isolated from embryonated eggs inoculated with a rabbit tick pool (Haemaphysalis leporispalustris), as reported by Clark in 1964 (3). Clark and Rourke (5) have reviewed the characteristics of the SMCA strain and a second related strain (GT-48) recovered from rabbit ticks a year after isolation of SMCA. A third strain of S. mirum (TP-2) was recovered directly in SP-4 broth medium from a rabbit tick pool collected in Maryland in 1977 (15). Several other spiroplasmas, serologically distinct from S. mirum, have been recovered from ticks, including the 277F strain from rabbit ticks (2) and the Y32 group from Ixodes ticks (16). The spiroplasmod natural of SMCA and GT-48 strains was conclusively established in 1976 by Tully et al. (18), and organisms were cultivated for the first time in a cell-free medium, SP-4, in 1977 (17). S. mirum killed 7-day-old embryonated chicken eggs during an incubation period of 4 to 10 days. Intracerebral inoculation of S. mirum into suckling mice, rats, rabbits, and hamsters produces cataracts, encephalitis, and death (3, 5, 9, 10). The specific effect depends on the medium used to propagate the organism, the animal species strain, and the number of organisms injected (7). The GT-48 strain, antigenically related to S. mirum, produces more severe pathogenic effects which generally result in overwhelming encephalitis, killing sucking animals within 7 days. Strain 277F did not produce cataracts in intracerebrally inoculated rats. It did kill sucking rats intracerebrally inoculated in high doses, >10^9 organisms. Strain 277F did not kill embryonated eggs (2).

Initial attempts were made to cultivate S. mirum in cell culture but were unsuccessful for cytopathic effects as well as for multiplication of the organism (1, 3). Chick embryo chorioallantoic membrane and yolk sac fragment cultures were unsuccessful (3). Organ culture system, using whole rabbit lens has been successful (6). In this study, Fabiyi et al. propagated uncloned S. mirum to obtain a 10^7.8 50% eye lens dose per lens culture. Steiner et al. (14) grew S. mirum in Drosophila DM-1 cells and obtained a cytopathic effect when incubation was made at 30°C but not when it was made at 25°C. At 25°C, S. mirum

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established a chronic infection of Dm-1 cells. The infected culture could be passaged; concentrations of ca. 10^6 color change units (CCU) of S. mirum were obtained per ml within 7 days.

The availability of a differentiated lens cell line from the rabbit, a host susceptible to S. mirum, encouraged us to examine S. mirum in this cell culture system to determine spiroplasma-eukaryotic cell interactions. Since cataracts are a major consequence of S. mirum infection, an appropriate cell culture model might help explain mechanisms of pathogenesis.

**MATERIALS AND METHODS**

Organisms. S. mirum strain SMCA was kindly provided in lyophilized form by J. G. Tully of the National Institute of Allergy and Infectious Diseases, Bethesda, Md. The strain was grown in SP-4 broth medium (17). All experiments were performed with a single stock supply of 10^6 CCU/ml. Samples (1 ml) of the stock were frozen at –70°C. These samples were quick thawed for individual experiments. The passage number of the organism at the beginning of these studies was ca. 100. Strain GT-48 (CMRL P3) passage 9 was also used and stored under the same conditions.

Cells. Two rabbit lens epithelial cell lines were used. These were established by Reddan et al. from 5-day-old rabbit (AG-4676) and 18-month-old rabbit (AG-4677) cell cultures. These have been characterized previously (12). They were obtained from the Aging Cell Repository, Institute for Medical Research, Camden, N.J.

A rabbit fibroblast cell line (RAB 9) passage 20 was obtained from the American Type Culture Collection (CCL 1414). A human fibroblast cell line (MRC-5) and the continuous mouse cell line 3T6 were obtained from departmental stocks. Medium for AG-4676 and AG-4677 was Dulbecco modified Eagle Earle medium (DMEM) 10% fetal bovine serum (FBS). RAB-9 and MRC-5 cells were grown in Ham F-12–10% FBS and McCoy–20% FBS, respectively. The cells were assayed for mycoplasma at the beginning and end of these experiments by microbiological culture and fluorescent DNA staining to ensure absence of accidental mycoplasmal infection (11).

**Spiroplasma growth and compatibility in cell cultures.** The growth curve of S. mirum in cell cultures was determined by inoculation of 10^4 CCU in 0.1-ml samples in 1 ml of cell culture medium after cell attachment with incubation at 37°C in 5% CO_2–air. Serial dilutions of this culture were made daily by the addition of 0.5 ml of cell-spiroplasma suspension to 4.5 ml of SP-4 medium. The number of viable organisms was made by determination of the number of CCU observed weekly for 1 month. Growth was defined as an increase of 2 logs in 6 days or less. Compatibility of S. mirum in different cell culture media was determined by dark-field microscopic examination for S. mirum motility, growth, and helicity.

**Cell culture growth curve.** Cell culture growth curves of AG-4676 or AG-4677 were performed in 25-cm² flasks by inoculation of 10^5 cells in 6 ml of medium. Three flasks were counted daily in triplicate with a hemacytometer: viability was calculated by using trypan blue. All growth curves were performed at least twice.

**Preparation of Spiroplasma extract.** S. mirum was grown in SP-4 medium for 7 days. The suspension was centrifuged for 30 min at 15,000 rpm in a Beckman type 30 rotor. The supernatant was filtered through a 0.22-μm filter and tested for toxic effects on cell cultures by inoculation of 0.1-ml samples onto monolayers of AG-4676 cells. A sonicate was prepared from the centrifuged pellet to assay for a nondiffusible toxic material as performed above for supernatant testing.

**Growth factor studies.** AG-4676 was grown in DMEM–10% FBS for 5 days or as described above. The supernatant medium was centrifuged at 500 × g in an International centrifuge for 15 min. The supernatant was successively filtered through 0.45- and 0.22-μm membrane filters. This cell-free-conditioned medium was used to test growth promotion of S. mirum that had been grown in SP-4 under various test conditions. All serial dilutions of S. mirum inocula were made in the conditioned medium to minimize effects of SP-4 nutrient carry-over. S. mirum (10^6 CCU) was used as inoculum into the conditioned medium.

**Animal studies.** Newborn Wistar rats were injected intracerebrally within 48 h of birth with 0.03 ml of undiluted S. mirum culture (ca. 10^9 CCU). Each mother and her litter were kept together in a separate cage throughout the study. The number of CCU was determined for each injection by inoculation of serial dilutions into SP-4 broth.

Karyology was performed on uninfected and S. mirum-infected AG-4676 by standard methods (19). Karyotypes were performed on 5-day-old cultures. For infected cultures, 10^6 CCU of S. mirum was added at the time of culture passage; karyotypes were performed 5 days later.

**RESULTS**

Among the different cell culture media tested, the most effective for growth of S. mirum in the cell cultures tested was DMEM. FBS which was not inactivated gave better results than inactivated FBS. Criteria for this evaluation was absence of cytopathology of cultured cells, maintenance of helical morphology of S. mirum for a minimum of 24 h, and an increase in S. mirum numbers. Rabbit serum, inactivated and not inactivated, resulted in poorer growth of AG-4676.

**Growth of S. mirum in AG-4676 and AG-4677 and effects of growth.** Rapid growth of S. mirum was observed in AG-4676 cell cultures compared to SP-4 broth medium. S. mirum did not grow in cell-free DMEM–10% FBS medium, indicating the need for cultured cells for growth (Fig. 1). Serial dilutions of S. mirum showed that 10^3 to 10^2 CCU could establish infection of AG-4676 cell cultures, growing to 10^7 to 10^8 CCU/ml in 7 days in different experiments.

Dark-field microscopy showed numerous spiroplasmas in the supernatant, but few were attached to the cells. This finding of few organisms attached to AG-4676 cells was confirmed.
by DNA fluorescent staining. In many instances, it was difficult to distinguish *S. mirum* from fluorescent artifacts seen in uninfected cells.

Light microscope observation of infected AG-4676 cells after 4 to 5 days showed numerous rounded cells floating in the medium and granulation and vacuolization of the cytoplasm. Giemsa staining showed an increase in size of the cells in relation to the control as well as an increase in the number of bi- and polynuclear cells (36/100 versus 14/100, *P* < 0.001) (Fig. 2). *Spiroplasma* sp. GT-48 also grew to high titers and produced cytopathology in AG-4676 cells.

No difference was noted in the karyology between infected and noninfected AG-4676. However, both cultures exhibited an extra band in chromosome 10 (Fig. 3). This was not observed in the original text describing this cell line (12).

Tests for a spiroplasmal toxin were negative when culture supernatants and sonicates were used, indicating the lack of a demonstrable toxin of *S. mirum* for AG-4676 under these conditions.

The effect of *S. mirum* on the growth of AG-4676 cells is presented in Fig. 4. AG-4676 cells grew relatively slowly, reaching confluency after ca. 7 days of incubation. Although *S. mirum* reached peak titers in 5 to 7 days in SP-4 broth medium, it reached peak titers in AG-4676 cells much faster, i.e., 2 to 3 days. *S. mirum* produced the greatest inhibiting effect late in the growth of AG-4676 cells. Growth of *S. mirum*-free AG-4676 cultures plateaued at 7 days; 7- to 11-day-old cultures had viabilities of 88 to 94%, similar to younger cultures. This was similar to the report of Reddan et al. for this cell line (12).

**Growth curve of *S. mirum* in other cell lines.** *S. mirum* also grew in RAB 9 and MRC-5 fibroblasts, but no cytopathic effect was noticed during the 1-week incubation period. *S. mirum* also grew in the mouse 3T6 cells. No cytopathology was evident after four passages in 3T6, even though *S. mirum* concentrations in the supernatant reached ca. 10⁸ CCU/ml.

**Growth in AG-4676 cell culture-conditioned medium.** When 10⁵ CCU of *S. mirum* were inoculated into the cell-free conditioned medium, rapid growth was evidenced. As shown in Fig. 5, growth was more rapid in the conditioned medium than in the SP-4 medium, with a much shorter lag period in the conditioned medium. No significant difference was noted in the peak concentrations obtained in the conditioned medium compared with SP-4. Conditioned medium prepared from RAB-9 and MRC-5 cells yielded the same results as that prepared from AG-4676 cells, indicating that the growth promotion was not a specific characteristic of the rabbit lens culture. Initial attempts to isolate a specific growth factor were unsuccessful. Conditioned medium from 1- and 5-day-old cultures yielded similar results. Further characterization of the conditioned medium was performed with media from 5-day-old AG-4676 cells. Initial characterization of the conditioned medium is shown in Table 1.

To determine whether the growth promotion of the conditioned medium could be due to osmotic conditioning of the DMEM–10% FBS medium, osmolarities were measured. No significant differences in osmolarity were observed among DMEM–10% FBS, conditioned medium from AG-4676 culture, or spiroplasmal SP-4 medium (330 mosM). In fact, adjustment of DMEM–10% FBS to 322 mosM did not promote growth of *S. mirum* (Table 1).

AG-4676-conditioned medium was tested for

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**FIG. 1.** Growth of *S. mirum* in AG-4676 cell cultures. SP-4 spiroplasma medium and DMEM–10% FBS, medium for AG-4676.
its ability to promote the growth of other spiroplasmas. *Spiroplasma* sp. GT-48, serologically and genetically related to *S. mirum*, grew in conditioned medium, similar to *S. mirum*. Conditioned medium failed to promote the growth of three other spiroplasmas tested, *S. citri*, corn stunt spiroplasma, and the sex ratio organism.

**Pathogenicity of cell culture-propagated *S. mirum* for suckling rats.** Intracerebral inoculation of suckling rats with *S. mirum* grown in

FIG. 2. Giemsa-stained preparation of *S. mirum*-infected AG-4676 cell culture. a. Uninfected; b. infected with *S. mirum*. 
either SP-4 or AG-4676 showed that \textit{S. mirum} maintained its pathogenicity for these animals after 10 passages in AG-4676 cells, still producing death and cataracts. No difference in cataract formation or animal death was detected between AG-4676-propagated (55/60, 91.7%) or SP-4-propagated (60/65, 92.3%) \textit{S. mirum}. Small numbers of animals died from each of three negative control groups (8/95) inoculated with SP-4 medium, DMEM–10% FBS, or AG-4676 cells in DMEM–10% FBS. Cataracts were produced in five rats injected with AG-4676-passaged material and seven rats injected with SP-4-passaged \textit{S. mirum}.

**DISCUSSION**

The effects of \textit{S. mirum} on rat and mouse eye tissues have been reported by Clark and Karzon (4) and Friedlander et al. (7), respectively. Similarities have been reported between rat and mouse \textit{S. mirum}-induced eye disease (5). In Sprague-Dawley rats, lens changes began ca. 5 days after injection. Lens epithelium proliferated, producing aberrant swollen and nucleated fibers central to the proliferated equatorial epithelium. A continuous band of multilayered epithelium eventually surrounded the lens as internal lens degeneration intensified (5).
Rabbits developed severe keratoconjunctivitis leading to pannus and intense inflammatory response in the anterior chamber (3). Kirchhoff et al. (9) reported lens changes in the rabbits consisting of fiber degeneration, liquefaction, inflammation, mineralization, fibrosis, and foreign body granulomatous reactions.

Lens epithelial cell culture is an appropriate in vitro system since lenses in vivo contain no blood supply, are not innervated, are completely encapsulated, and are derived entirely from ectoderm (12). S. mirum grew to high titers of $10^8$ CCU/ml of supernatant medium from AG-4676 cultures. Interestingly, the organism did not grow in cell culture medium, indicating the need of cells or a cellular product for growth. More studies are required to determine the relative value of this and perhaps other lens cell lines as in vitro models for S. mirum. As shown in this study, the organism grows to high titers in other cells which are not lens or rabbit cells. Clearly, however, these studies demonstrate the potential value of cell and organ culture systems to study spiroplasmal pathogenicity. For years, attempts to propagate S. mirum in cell-free medium were unsuccessful until Tully et al. developed SP-4 medium (17). Interestingly, Fabiyi et al. (6) previously clearly demonstrated that S. mirum consistently grew to high titers in rabbit eye lens organ cultures. For some reason, this study was not further developed, although the value of the system was clearly apparent.

In a short study, Kirchhoff et al. (9) noted changes in the lens epithelium of S. mirum-infected rabbits, namely degeneration of lens fibers, and liquefaction of lens substance. The expression of lens fibers and lens substance are not precisely known in the AG-4676 cell line. Reddan et al. stated that the line expressed

Clark and Karzon (4) reported similar findings in C57BL/6Ha mice, the most susceptible strain of six tested. S. mirum was first detected in retinal tissue 2 days after injection and then in the lens (day 4), chorioid and globe (day 7), and in the cornea and anterior uveat tissues (day 7 to 10). Because infection and pathological changes in the retina consistently preceded lens effects, Clarke and Rourke have noted that S. mirum-induced cataracts may be secondary cataracts (5).

![Figure 4](http://iai.asm.org/) Effect of S. mirum on growth of AG-4676 cell cultures. Symbols: ○, AG-4676 with S. mirum; ★, AG-4676.

![Figure 5](http://iai.asm.org/) Growth of S. mirum in cell-free conditioned medium of AG-4676. Symbols: ○, Conditioned medium; ★, SP-4 medium.
TABLE 1. Characteristics of growth promotion of S. mirum by conditioned medium from AG-4676 cell cultures

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Growth promotion</th>
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<tr>
<td>DMEM–10% FBS (AG-4676 medium) (292 mosM)</td>
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</tr>
<tr>
<td>DMEM–10% FBS (322 mosM)</td>
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<tr>
<td>Conditioned medium</td>
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<tr>
<td>None (control)</td>
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<td>Heat, 56°C, 30 min</td>
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<tr>
<td>Freeze-thaw</td>
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<td>Filtration, 0.220 µm</td>
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<tr>
<td>Dilution</td>
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<td>1:10</td>
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rabbit crystallins, but this was not completely proven (12). More data are needed on the degree of differentiation of this cell culture.

One advantage of the AG-4676 cell culture system is that this culture grows relatively slowly, reaching confluence

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SP-4. As noted, S. mirum grew more rapidly in AG-4676 than in SP-4. AG-4676 cells were stable and viable for periods after reaching confluency. In fact, this postconfluence period may represent a more differentiated form of the culture and be more important for the study of S. mirum.

Although no toxin could be demonstrated in S. mirum, cell-free conditioned medium from AG-4676 cultures promoted growth to approximately the same extent as SP-4 medium, but with no appreciable lag period. The nature of this growth promotion is unknown and under investigation. It does not appear to be due to osmotic conditioning of the medium, since the conditioned medium had a similar osmolarity to SP-4 medium, and adjustment of DMEM–10% FBS to 322 mosM failed to promote S. mirum growth.

The effects of medium osmolarity on growth of S. mirum merits investigation. S. mirum can grow in SP-4 medium (332 mosM), conditioned medium (292 mosM), Schneider Drosophila cell culture medium (318 mosM), and spiroplasmal M1A medium (534 mosM) (values obtained in this laboratory). The growth of S. mirum over such a wide range of osmolarities is of interest in view of its original isolation from a tick and its ability to produce disease under appropriate conditions in certain animals. Tick hemolymph has a high osmolarity.

Apparently, a cellular product elaborated early in the growth of AG-4676 cultures and other mammalian cultures promoted S. mirum growth, even faster than SP-4 medium designed specifically for S. mirum (17). Studies are in progress to attempt to characterize the nature of growth promotion by the conditioned medium by using standard methods (8, 13). The substance may or may not be a component of SP-4 medium. In either case, its identification may add to our knowledge of S. mirum metabolism and result in the development of new or modified media for S. mirum.

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LITERATURE CITED


