Pathogenic Mycoplasmas: Cultivation and Vertebrate Pathogenicity of a New Spiroplasma

Abstract. *A spiroplasma recovered from allantoic fluids of chick embryos infected with the tick-derived suckling mouse cataract agent was grown in continuous passage on a new artificial culture medium. The cultured organisms induced typical ocular and other disease symptoms in susceptible animals, and were reisolated from involved host tissues. Although spiroplasmas have been previously recognized as plant and insect pathogens, this is the first spiroplasma shown to multiply at 37°C and to be pathogenic for vertebrates.*

Spiroplasmas (*1*) are helical, motile mycoplasmas (class Mollicutes). In 1971, a mycoplasma that caused symptoms of stubborn disease in citrus was cultivated (*2*) and was subsequently identified as a spiroplasma by morphological (*3*) and biological (*4*) criteria. An isolate of this organism (*Spiroplasma citri*) from Israel was subsequently shown to induce symptoms typical of citrus little leaf disease (*5*). In 1975, two independent groups of investigators cultivated the corn stunt organism (CSO) and demonstrated its pathogenicity for corn (*6*). A third possible spiroplasma [sex-ratio organism (SRO)] is vertically transmitted in four closely related species of *Drosophila*, but has not been cultivated nor shown to be associated with plants (*7, 8*). Thus, spiroplasmas were regarded as plant or insect microorganisms until we recently reported (*9*) the association of a new spiroplasma with a disease syndrome experimentally induced in rodents by an infectious, egg-passaged entity termed the suckling mouse cataract agent (SMCA). This filterable organism, first isolated (*10*) from a pool of rabbit ticks (*Haemaphysalis leporispalustris*), was initially thought to resemble slow viruses (*11*). However, subsequent ultrastructural studies (*12*) suggested that the agent might be a mycoplasma, although it could not be grown on conventional mycoplasma media or in cultured cells (*10*). We now report the cultivation of SMCA on artificial medium and document the ability of cultured organisms to induce classical disease symptoms in animal hosts.

In our first attempts to cultivate SMCA and another (presumably similar) tick-derived isolate (GT-48) we added portions of infected chick embryo allantoic fluid either to a variety of conventional liquid and solid mycoplasma media (*13*), or to medium formulations used for the primary isolation of *S. citri* (*2*) or CSO (*6*). However, all such media, whether or not blind passages were made from them, failed to support SMCA growth at temperatures ranging from 30° to 37°C. A concurrent study by Jones *et al.* (*14*) that assessed the importance of medium components for the growth of plant spiroplasmas suggested a rationale for new, alternative formulations. Eighteen of these new formulations were prepared, at least two of which (SP-1 and SP-2) (Table 1) supported the growth of SMCA and GT-48 isolates after addition of 1 ml of infected allantoic fluid to 7 ml of medium. Cultures appeared turbid, and a slight acidic shift in *p*H occurred after 10 to 14 days of incubation at either 30° or 37°C. Examination of such cultures by dark-field microscopy (× 1250) revealed large numbers of motile, helical organisms. After several continuous passages, peak titers of 10^8 to 10^9 color-changing units per milliliter (based on changes in phenol red indicator in serial tenfold dilutions of culture) were recorded after 5 to 7 days of incubation. The slightly modified medium (SP-4) (Table 1) that we now use for primary isolation and maintenance of SMCA and GT-48 strains gives an isolation rate of 100 percent when 0.1-ml portions of infected allantoic fluid (from continuous egg-passaged lines) are added to 2 ml of medium.

The morphology of cultured SMCA and GT-48 organisms was similar to that observed earlier (*9*) with egg-passaged organisms. Electron micrographs of negatively stained cells revealed the classical helical shape (Fig. 1A), and examination of thin-sectioned pellets (Fig. 1B) confirmed the absence of structures such as cell wall and axial filaments which distinguish spiroplasmas from spirochetes and other true bacteria.

Strains SMCA and GT-48 produced colonies on 3.5 percent Noble agar plates prepared from either SP-1 or SP-4 media (Table 1). Colonies were visible after 14 to 17 days of incubation at 30°C on plates placed in an atmosphere of 95 percent N_2 and 5 percent CO_2. Aerobic incubation prolonged but did not inhibit the appearance of colonies; agar concentrations of less than 3 percent were unsatisfactory for good colony formation. Classical "fried egg" colonies did not develop on these media. Typical colonies were somewhat diffuse and irregular, and much of their growth occurred within the agar (Fig. 1C). Nevertheless, viable cells could be enumerated by counting the colonies. For example, a count of 2×10^9 colony-forming units per milliliter was obtained by plating an 8-day culture (30°C) of SMCA from SP-4 broth medium.

The development of solid growth media also provided a means for culture purification in accordance with published requirements (*15*) for description of new members of the order Mycoplasmatales. The SMCA and GT-48 isolates were triply cloned by ultrafiltration of late log-phase liquid cultures (5 to 7 days) through membrane filters having an average pore diameter of 220 nm, and subsequent plating of serial tenfold dilutions. Reduction in titer of the two isolates was usually less than three $\log_{10}$

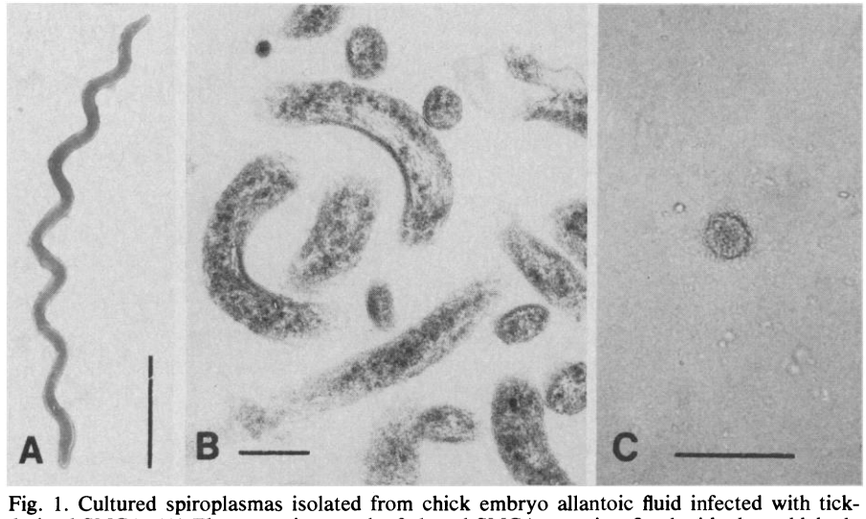

Fig. 1. Cultured spiroplasmas isolated from chick embryo allantoic fluid infected with tick-derived SMCA. (A) Electron micrograph of cloned SMCA organism fixed with glutaraldehyde and stained with 1 percent aqueous uranyl acetate. Scale bar, 0.5 μm. (B) Electron micrograph of thin section of pellet of cloned SMCA. Scale bar, 0.11 μm. (C) SMCA colony. Scale bar, 0.1 mm.

colony-forming units per milliliter. Triply cloned cultures, which represent about 25 to 30 continuous passages on artificial medium, were deposited in the American Type Culture Collection, Rockville, Maryland (SMCA: ATCC 29335) (GT-48: ATCC 29334).

Serological assessment of the relationship of the SMCA and GT-48 isolates with other plant and insect spiroplasmas was performed as previously outlined (*8, 16, 17*). These tests (Table 2) established that SMCA and GT-48 are closely related or serologically identical members of a cluster of spiroplasmas which are partially related to CSO and *S. citri*. The deformation test (*8*) appeared to be especially suitable for distinguishing cultivable spiroplasmas. Sharing of major antigenic components by various plant and insect spiroplasmas has been demonstrated in a number of careful serological studies (*6, 8, 16*). Since antigenic sharing of this magnitude is uncharacteristic of mycoplasma species, previous investigators (*6*) have been reluctant to assign taxonomic epithets to other newly isolated spiroplasmas. We agree with this approach and prefer to designate SMCA simply as a new spiroplasma strain until sufficient information is available to establish its true taxonomic status.

It was deemed essential to demonstrate that pure cultures of SMCA and GT-48 spiroplasmas could induce classical vertebrate pathogenicity (*10*) in embryonated hen's eggs, or in suckling rats and mice. Egg-passaged as well as cultured (in liquid broth) organisms of each SMCA isolate, when assayed for infectivity in 7-day-old chick embryos, gave 50 percent egg lethal dose (ELD_{50}) titers ranging from 8.0 to 9.5 $\log_{10}$ per milliliter. Egg-passaged SMCA characteristically induces cataracts, uveitis, and chronic brain infection in suckling mice and rats after they are challenged intracerebrally at early ages, usually less than 96 hours after birth (*10*). Although cataract incidence in suckling rats of the Wistar or Sprague-Dawley lines is usually 100 percent, the occurrence of cataracts in mice varies from 3 to 85 percent, depending upon mouse strain (*10*). The responses of suckling mice and rats to intracerebral challenge with cultured (in liquid broth) SMCA or GT-48 isolates were similar in most respects to responses observed after challenge with egg-passaged organisms. Approximately 50 to 60 percent of mice or rats receiving undiluted SMCA cultures died within 5 to 10 days after challenge. Nearly all (98 to 100 percent) suckling rats (Sprague-Dawley) that survived inoculation with the cloned SMCA culture exhibited bilateral cataracts (Fig. 2). However, surviving mice (ICR strain), unlike those challenged in earlier studies with egg-cultivated SMCA, did not develop cataracts. Although intracerebrally inoculated, egg-cultivated GT-48 is known to induce higher mortality (100 percent) in both suckling mice and rats than the SMCA isolate, there is no evidence at present that GT-48 can induce cataracts in either host (*10*). The

Table 1. Composition of growth medium for SMCA spiroplasmas. Basal medium was adjusted to *p*H 7.5 and then autoclaved at 121°C for 15 minutes. Complete medium was prepared by the addition of each of the sterile supplements. Agar plates were prepared, generally with 200-ml amounts of the complete medium, by the addition of 3.5 percent (weight per volume) of Noble agar (Difco) to the basal medium before sterilization. After autoclaving, the basal medium was equilibrated at 56°C, sterile supplements were added (except the phenol red solution), and the plates poured. The agar medium was used no more than 7 days after preparation. The SP-2 medium contains the same ingredients as SP-1, except that the CMRL 1066 supplement was replaced with an equal volume of Medium 199 (10 ×) (minimal essential medium with Earle's salts and glutamine) (Gibco 118E). The SP-4 medium in current use consists of SP-1 components but lacks α-ketoglutaric acid and contains an additional 50 ml of deionized water in the basal medium. Abbreviations: BBL, Baltimore Biological Laboratories; Difco, Difco Laboratories; Gibco, Grand Island Biological; MA, Microbiological Associates; Flow, Flow Laboratories.

Ingredient	SP-1 medium (amounts per 1 liter)
Basal medium	
Mycoplasma broth base (BBL)	3.5 g
Tryptone (Difco)	10.0 g
Peptone (Difco)	5.3 g
Glucose	5.0 g
Deionized water	565.0 ml
Supplements	
CMRL 1066 medium (10 ×) (with glutamine) (Gibco 154)	50 ml
α-Ketoglutaric acid (0.4 percent solution, sterile)	50 ml
Fresh yeast extract (25 percent solution) (MA)	35 ml
Yeastolate (2 percent solution, sterile) (Difco)	100 ml
Fetal bovine serum (heated at 56°C for 1 hour) (Flow)	170 ml
Penicillin (100,000 unit/ml)	10 ml
Phenol red solution (0.1 percent solution)	20 ml
Osmolality, 332 mosmol; *p*H 7.0 to 7.4	

Table 2. Serological relationships of SMCA and GT-48 spiroplasmas to other spiroplasmas. Antibodies to triply cloned organisms were produced in each of two rabbits by techniques described earlier (*16*). Results of the disk growth inhibition test (*16*) are expressed as zones of inhibition (in millimeters) around paper disks impregnated with undiluted antiserum. Deformation (*8*), precipitin ring (*16*), and complement fixation (*17*) test results are expressed as reciprocals of dilution end points of each test. Pooled serum from rabbits prior to immunization gave maximum zones in growth inhibition tests of ≤ 1 mm and maximum titers of 40, 2, and 64 for deformation, precipitin ring, and complement fixation tests, respectively. Abbreviation: CSO, corn stunt organism.

Antigen	Antiserum to			
	S. citri (Morocco)	CSO (Rio Grande)	SMCA	GT-48
	Growth inhibition test			
Morocco	13	10	0	1
CSO	13	>18	1	2
SMCA	0	0	16	16
GT-48	0	0	18	18
	Deformation			
Morocco	> 10,240	< 20	< 20	< 20
CSO	80	1,280	< 20	< 20
SMCA	20	20	2,560	5,120
GT-48	< 20	20	1,280	2,560
	Precipitin ring			
Morocco	128	64	8	4
CSO	64	128	8	4
SMCA	32	16	128	128
GT-48	32	16	128	128
	Complement fixation			
Morocco	8,000	1,000	160	40
CSO	1,000	16,000	640	640
SMCA	320	640	2,000	8,000
GT-48	80	80	2,000	8,000

GT-48 strain cultured on artificial medium and then cloned was also highly lethal to suckling rats but produced no cataracts in the survivors (*18*) receiving cultures diluted from 10^{-2} to 10^{-5}.

We confirmed the association between spiroplasmas and the classical pathogenicity in animals by recovering organisms from infected tissues and demonstrating that the recovered organisms were serologically related to, or identical with, those employed in challenge. Twenty-nine rats exhibiting cataracts were autopsied 3 to 4 weeks after intracerebral challenge with cultured SMCA (30th passage in media). Brain and eye tissue pools were cultured separately in SP-4 medium. Ten rats that had received uninoculated culture medium intracerebrally were examined in the same way. Spiroplasmas were recovered from the brain and eye pool of all infected rats, but never from control rat tissues. At least ten isolates, recovered from the eye and brain, were examined in the deformation test and shown to be serologically indistinguishable from SMCA. Thus, our pathogenicity experiments establish that both cultured and cloned SMCA and GT-48 spiroplasmas are able to induce disease manifestations typical of those initially described (*10*), and that recoverable organisms indistinguishable from the challenge organisms persist in the tissues of affected rodents.

The natural reservoir of SMCA spiroplasmas is unknown. However, the recovery of two isolates from rabbit ticks (*10*), the reported recovery of a third, possibly similar, organism from the same tick species (*19*), and the demonstrated persistence of the agent in animal tissues suggest that SMCA might be maintained in a tick-vertebrate cycle in nature. Certainly, an affinity for arthropods is at present a common link for all known spiroplasmas.

The ability of SMCA to multiply over a higher temperature range (30° to 37°C) distinguishes it from all known plant and insect spiroplasmas, which have an optimal temperature range of 25° to 32°C and fail to grow at temperatures above 34°C. Our observation that SMCA could be isolated only on a specially formulated medium explains previous failures to cultivate the agent. The inability of a variety of standard artificial media and cell cultures to support growth of SMCA provides a strong indication that other unrecognized mycoplasmas, including perhaps human or animal pathogens, await discovery. Thus, these organisms represent a new class of microbial agent, incapable of being cultured on the usual laboratory medium and undetected by current serological procedures, yet possessing the ability to provoke disease in vertebrates and to persist for prolonged periods in host tissue. We believe a continued search for the agent in human and arthropod hosts, and for development of information on their possible role in human diseases of unknown etiology would seem worthwhile.

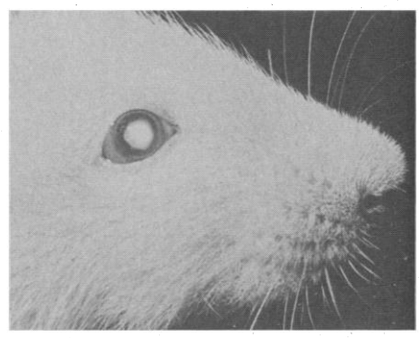

Fig. 2. Rat, 25 days old, with severe bilateral cataracts following intracerebral inoculation as newborn (24 hours) with cloned SMCA passaged 26 times on artificial medium.

JOSEPH G. TULLY
Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20014
ROBERT F. WHITCOMB
Plant Protection Institute, Agriculture Research Service, U.S. Department of Agriculture, Beltsville, Maryland 20705
H FRED CLARK
Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania 19104
DAVID L. WILLIAMSON
Department of Anatomical Sciences, State University of New York, Stony Brook 11794

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18. Suckling rats (Sprague-Dawley) were challenged intracerebrally on day 2 after birth with 0.03 ml of an inoculum of the GT-48 strain (triply cloned line at the 6th to the 22nd passage level) diluted in broth from 10^{-2} to 10^{-5}. Mortality, recorded at 4 to 5 weeks, for challenge dilutions 10^{-2} through 10^{-5} was 100, 83, 76, and 65 percent, respectively (N = 20, 59, 58, and 60, respectively).
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20. We thank N. Ramsburg, D. Rose, C. Blood, and M. Coan for excellent technical assistance. We are grateful to L. Cline for the complement fixation tests.

10 September 1976; revised 26 October 1976

Vision in Insects: Pathways Possibly Underlying Neural Adaptation and Lateral Inhibition

Abstract. *Like horizontal cells in vertebrate retinas, horizontal amacrine cells beneath the insect eye intervene between receptors and interneurons at the first level of synapses. Synaptic arrangements between amacrines and interneurons that give rise to regular networks of axon collaterals may explain recent electrophysiological observations of lateral inhibition beneath the insect retina. Neural adaptation mechanisms acting on single retinotopic channels or assemblies of channels can also be referred to reciprocal relationships between receptors and first-order interneurons as well as to centrifugal cells from levels of so-called photopic receptor endings.*

In arthropods, lateral inhibition and neural adaptation mechanisms have been observed at the level of visual receptors or their endings. In *Limulus*, contrast enhancement has been proposed to be mediated by pathways between ommatidia (*1, 2*), and, in insects, lateral inhibitory processes have been referred to axon collaterals of peripheral neurons in the lamina's external plexiform layer (EPL) (*3, 4*). Also, recordings from EPL-derived interneurons revealed light adaptation phenomena which are similar to those in their possible vertebrate ana-