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Adaptation of *Mycoplasma hominis* to an Obligate Parasitic Existence in Monkey Kidney Cell Culture (BSC-1) (38821)

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(Introduced by Bernard A. Briody)

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Many cell cultures are contaminated with mycoplasmas or become infected on continued subculturing of the cells. Therefore, the routine monitoring of cell cultures for the presence of mycoplasmas is recommended.

During attempts to eliminate mycoplasmas from a contaminated BSC-1 cell line of monkey kidney cells, the cell culture medium was monitored routinely for the presence of mycoplasmas by inoculation of Eaton Agent agar. This paper reports that the mycoplasmas gradually ceased to grow on this medium although their presence in the cell cultures could be confirmed by electron microscopy (EM).

Materials and Methods. A BSC-1 cell line of monkey kidney cells was maintained which grew poorly and showed cytopathogenic effects (CPE) suggestive of mycoplasma contamination. The cell culture medium consisted of Eagles MEM, (Grand Island Biological Co., NY) 100 μg/ml streptomycin, 100 units/ml penicillin, 1% anti-PPLO agent (Gibco), and 10% heat-inactivated fetal calf serum (Gibco). The cells were grown at 37° C in 32-oz prescription bottles containing 40–50 ml medium.

Control of mycoplasma contamination. The infection of the BSC-1 cell culture with mycoplasmas was controlled by propagating the cells for approximately 1 mo alternately in medium in which either the anti-PPLO agent had been increased to 4% or in medium in which the anti-PPLO agent was replaced with an "antibiotic cocktail" containing kanamycin, novobiocin, and aureomycin (1).

Isolation and identification of the mycoplasmas. The mycoplasmas were isolated on modified Eaton agent media (2) which were prepared from the same batch of constituents. They consisted of either 3.4% PPLO agar or 2.1% PPLO broth (Difco, Detroit, MI) supplemented with 20% unheated horse serum (Tissue culture select, Baltimore Biological Laboratories), 10% yeast extract (3), and Eagles MEM vitamins (BBL). Broth cultures in screw-capped tubes were incubated in a water bath for 72 hr and agar plates in a humidified incubator gassed with 5–10% CO_2 in air for up to 21 days. Cultures were incubated at 37° C. The mycoplasmas were identified by the growth-inhibition test (4) in which discs impregnated with specific antiserum (Taxo discs, BBL) are placed on the surface of lawn plates seeded with a broth culture of the mycoplasma.

Electron microscopy. The cells were detached from the glass surface with a rubber policeman, centrifuged at 1000*g* for 20 min, and the medium decanted. The pellet was resuspended in 2% glutaraldehyde in 0.1 *M* cacodylate buffer and fixed for 30 min at room temperature. The fixed cells were centrifuged, the fixative decanted, and the pellet washed in Millonig's buffer (5). After postfixation for 1 hr in Millonig's buffer containing 1% osmic acid, the pellet was dehydrated in a graded series of ethanol before final dehydration in propylene oxide and embedding in epon (6). Thin sections were cut in the gray-to-silver interference color range, collected on Formvar-coated 200-mesh copper grids, stained for 5 min with a saturated solution of uranyl acetate in 50% ethanol, washed with distilled water, counterstained for 3 min with lead citrate, and the excess lead removed by washing (7). The stained sections on the grids were coated

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lightly with carbon and examined in a Hitachi HU-12 electron microscope with an accelerating voltage of 75 kv.

Results. On inoculating Eaton agent agar plates with drops of the supernatant medium of the BSC-1 cell line, a primary culture of "fried egg" colonies typical of classical mycoplasmas was observed after 3–4 days incubation. To obtain secondary mycoplasma cultures, a block of agar containing colonies was cut from the primary culture and inoculated into broth or placed colony side down and pushed across the surface of a sterile Eaton agent plate, i.e., "friction inoculated" (8). The primary cultures which formed colonies on agar in 3 days could not be subcultured on Eaton agent agar by friction inoculation. The Eaton agent broth which was inoculated from the primary agar cultures was incubated for 72 hr and then inoculated onto Eaton agent agar. Colonies formed which indicated that the mycoplasmas had grown in the broth. Therefore, some aliquots of the broth cultures were ampouled and stored at −70° C while others were used to prepare lawn plates for the inhibition test which indicated that the contaminant was *Mycoplasma hominis.*

During the early stages of the treatment of the contaminated culture with the antimycoplasma agents, *M. hominis* was isoloated. Thereafter, growth of the mycoplasmas diminished until they ceased to form colonies on Eaton agent agar. Moreover, the organisms would not grow on plates incubated anaerobically in a Gas Pak anaerobic jar (BBL), which is considered a more sensitive method for the isolation of mycoplasmas from cell cultures than a gassed incubator (9). This suggested either that the mycoplasmas had been eradicated from the cell culture or that the organisms had become more fastidious in their growth requirement. However, the continued poor growth and the morphology of the cells indicated that the culture still was infected with mycoplasmas. Therefore, an attempt was made to improve the Eaton agent agar by replacing the horse serum with 10% calf serum. Growth did not occur on this medium even though the mycoplasmas could be seen in the cell cultures by EM. Obviously, the fetal calf serum did not contain the required metabolite.

Electron microscopy of infected cells. To confirm by direct observation that the cell line was infected, cells were examined by EM and the mycoplasmas were identified easily in thin sections. The organisms were seen in close proximity to viable cells (Fig. 1), but seldom associated with vacuolated and necrotic cells. Mycoplasmas replicating by budding were observed frequently around viable cells (Fig. 1). Only a few cells contained mycoplasmas which were seen either in a vacuole bounded by a membrane (Fig. 2) or free in the cytoplasm (Fig. 3). Because none of these intracellular elementary bodies was observed producing buds, it was possible that the intracellular mycoplasmas were not viable. Mycoplasmas and viable cells often were seen touching each other and the membranes were contiguous. However, occasionally a sphere was seen which could be considered a mycoplasma budding from the cell membrane (Fig. 4). The membrane of the sphere was continuous with that of the cell (Fig. 4) and the sphere contained structures similar to ribosomes.

Storage at −70° C. Ampoules of broth cultures of *M. hominis* which grew on Eaton agent agar had been stored at −70° C. After 3 mo of storage the mycoplasmas could not be recovered on either Eaton agent agar or in Eaton agent broth. Thus, it was not possible to obtain cultures for antibiotic-sensitivity tests. The ampoules were stored with other cultures of *M. hominis* which have maintained their viability for several years. Therefore, this strain of *M. hominis* differs from the other strains not only in its fastidious growth requirements but also in other characteristics.

Discussion. During attempts to eradicate *Mycoplasma hominis* from a BSC-1 cell culture with antibiotics, the mycoplasma gradually failed to grow on Eaton agent agar but continued to cause CPE in the cell culture which suggested that the mycoplasma had adapted to grow only in the presence of viable cells. This observation differs from the suggestion that there is complete agreement between the inoculation of mycoplasma-free indicator cells and agar

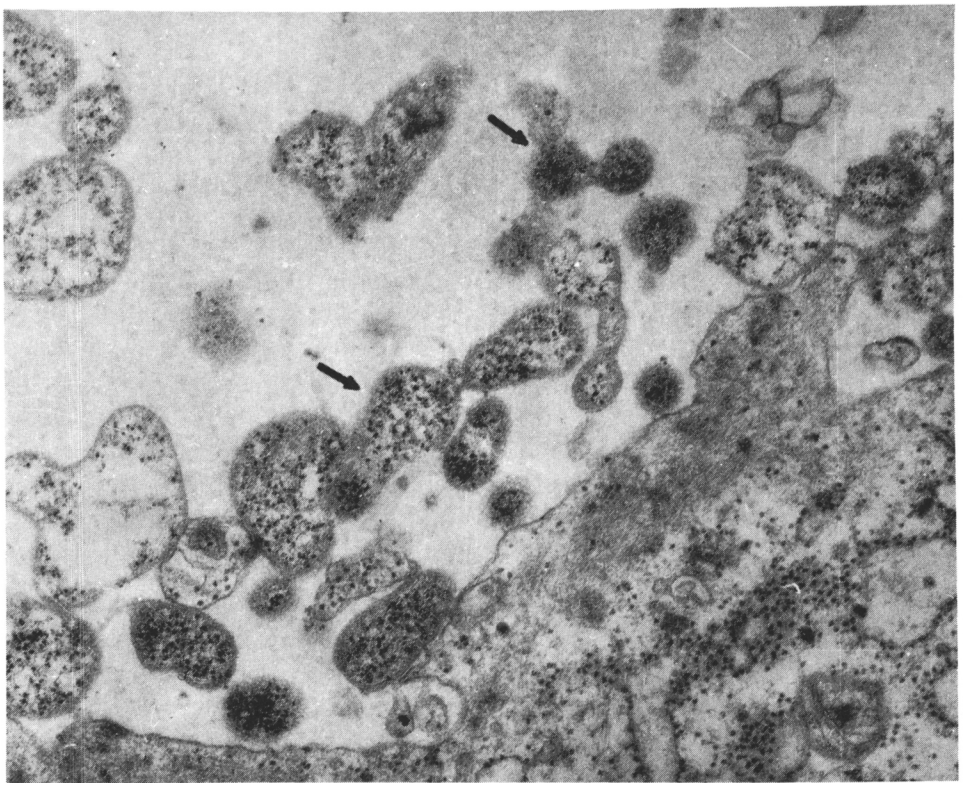

FIG. 1. Budding *Mycoplasma hominis* (arrows) near a viable BSC-1 monkey kidney cell. ×30,000.

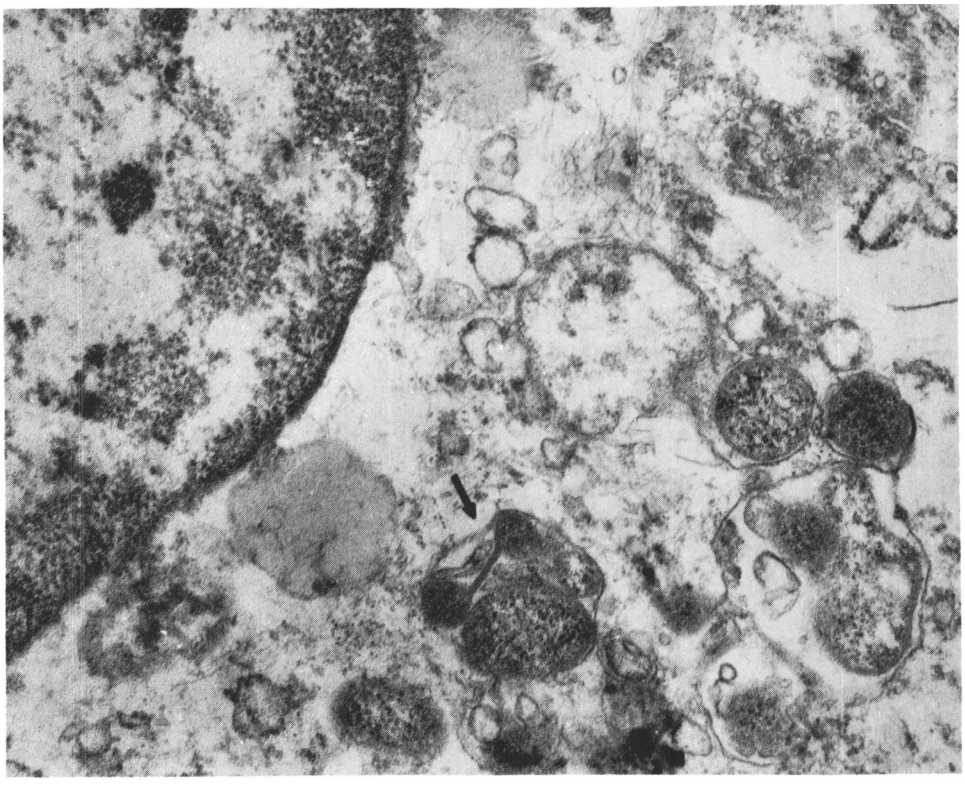

FIG. 2. *Mycoplasma hominis* (arrow) within a vacuole of a monkey kidney cell. ×30,000.

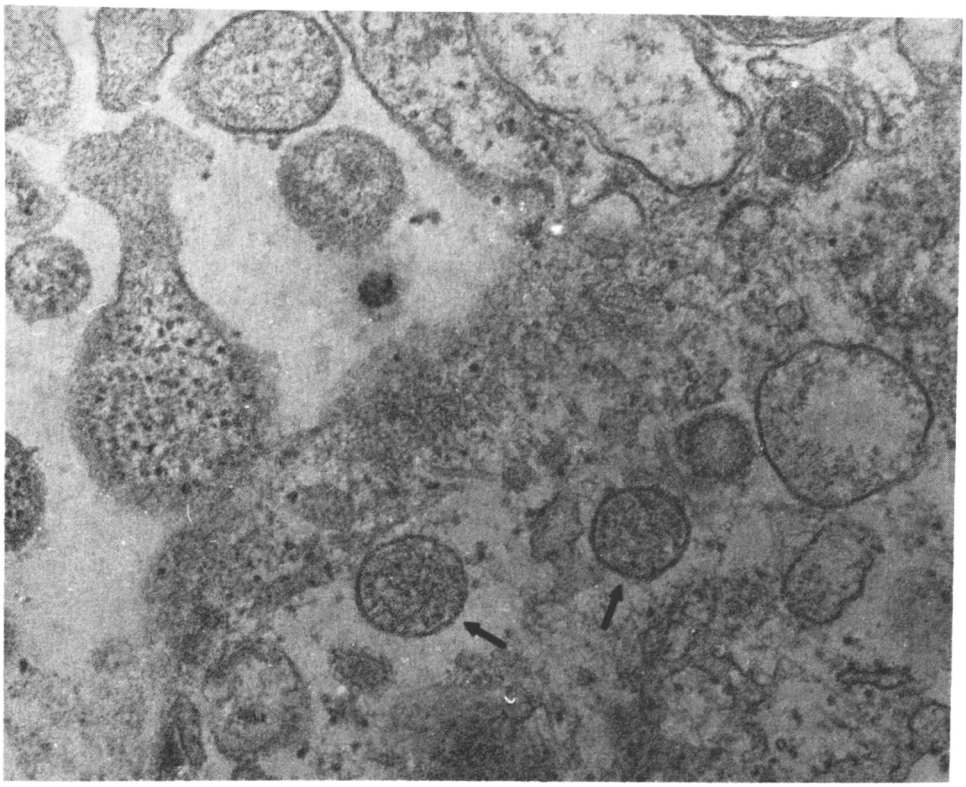

FIG. 3. Cytoplasm of a viable BSC-1 cell containing *Mycoplasma hominis* (arrows). ×30,000

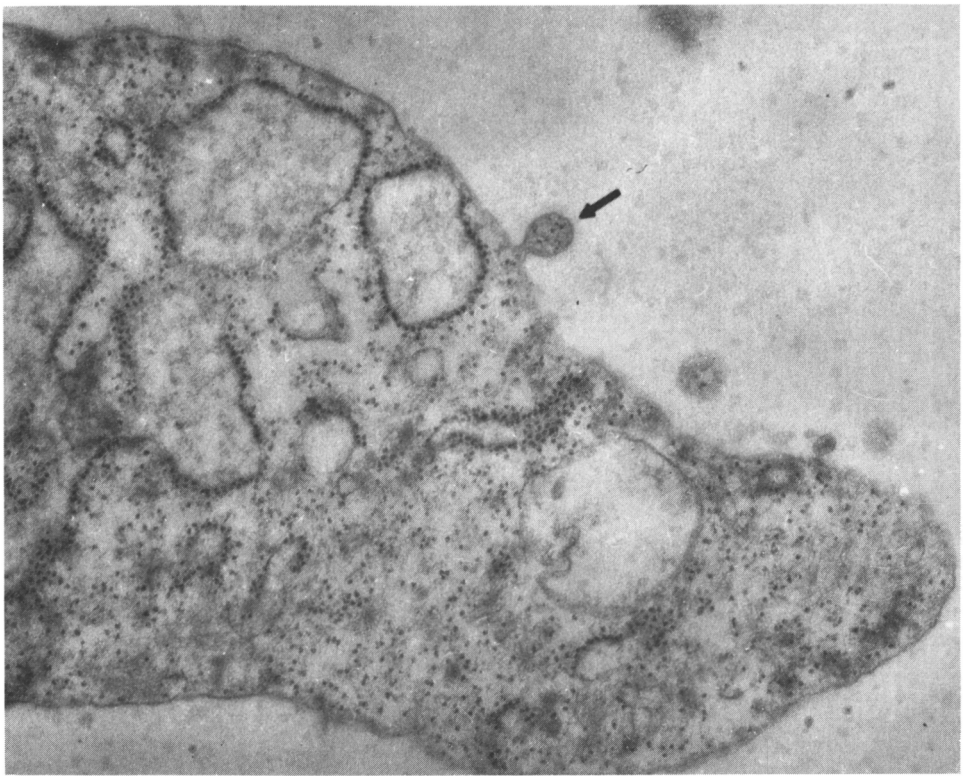

FIG. 4. Early stage of a *Mycoplasma hominis* budding from the cell membrane of a BSC-1 cell. ×30,000.

growth methods for the detection of mycoplasma contamination of cell cultures (10), and supports the reports that the inoculation of cell cultures is a more sensitive technique for detecting mycoplasma contamination (11). However, CPE in cells can be caused nonspecifically. Thus, the presence of mycoplasmas must be confirmed either by light microscopy (12, 13) or by EM (14). Though mycoplasmas which initially could be grown only in cell cultures have been adapted to grow on mycoplasma media (15), to our knowledge this is the first report of a mycoplasma which has adapted to a parasitic or symbiotic relationship with a mammalian cell culture.

Many techniques have been suggested for the eradication of mycoplasmas from cell cultures but are seldom successful. This strain of *M. hominis* was not eliminated by antibiotic treatment. However, the level of infection was reduced significantly by including antibiotics in the cell culture medium. Failure to eliminate the mycoplasmas with antibiotics may be explained by their mutation to antibiotic resistance. On the other hand, the mycoplasmas may resemble some intracellular parasitic bacteria which exist within phagocytic cells and are protected from a bacteriocidal or bacteriostatic concentration of the antibiotic in the body fluids when the cell membrane restricts the passage of antibiotics into the phagocytes.

Because the mycoplasmas ceased to grow on agar medium, it was not possible to ascertain whether this strain of *M. hominis* had mutated to antibiotic resistance or was being protected by the impermeability of the cell membrane to the antibiotic cocktail.

In cell cultures contaminated with mycoplasmas the viable cells not only are surrounded by the mycoplasmas but many cells contain large numbers of ingested elementary bodies (16). However, it has not been shown that the mycoplasmas divide within the cells. In electron micrographs of our infected BSC-1 cell line, many mycoplasmas were seen in close proximity to viable cells while only a few were observed near vacuolated and necrotic cells. *M. hominis* characteristically divides by budding (17) and budding cells were seen in the medium around the viable cells. No budding mycoplasmas were observed intracellularly but mycoplasma-like structures were seen either free in the cytoplasm or confined to vacuoles within the cell. The observed structures were considered mycoplasmas because they were bounded by a single trilaminar membrane, were spherical or oval, measuring 0.4 μm to 0.9 μm diam, contained ribosomes, DNA fibers and usually were vacuolated. Mycoplasmas can be distinguished from healthy mitochondria because the mitochondria of these monkey kidney cells tended to be more ovoid and elongated. Moreover, the mitochondria invariably contained discrete cristae radiating from the limiting membrane, which were not possessed by the mycoplasmas. The mycoplasmas differed from degenerating mitochondria which contain remnants of cristae or fragments of trilaminar membrane and show no evidence of fibrillar DNA. Also, mycoplasmas are differentiated from a section through an active ergastoplasm by means of the ribosomes attached to the external surface of the trilaminar membrane of the ergastoplasm. In contrast, the ribosomes of the mycoplasmas are free and are viewed within the trilaminar membrane. It was not known whether these intracellular mycoplasmas were viable until bud-like structures resembling mycoplasmas were observed protruding from the cell surface. The membrane of the bud-like structures was continuous with the cell membrane. Therefore, it is reasonable to postulate that some mycoplasmas can replicate within cells and then leave the cells by budding in a manner similar to the exokinesis of some viruses.

Summary. During attempts to eliminate *Mycoplasma hominis* from a monkey kidney BSC-1 cell line with antibiotics, the mycoplasmas were isolated repeatedly. However, the organisms ultimately failed to grow on medium although electron microscopy confirmed that the cell culture still contained mycoplasmas. Thus, the mycoplasma had adapted to an environment in which viable cells were required for growth. Budding mycoplasmas which are indicative of replication were seen associated with viable cells extracellularly. Moreover, structures resembling mycoplasmas were observed budding from the cells which suggests that the mycoplasmas replicate within the cells and

are similar to many viruses in their manner of release from the cells.

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