

# Cytopathogenic Mycoplasmas Associated with Two Human Tumors

## II. Morphological Aspects

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### ABSTRACT

HUMMELER, K. (The Children's Hospital of Philadelphia, Philadelphia, Pa.), D. ARMSTRONG, AND N. TOMASSINI. Cytopathogenic mycoplasmas associated with two human tumors. II. Morphological aspects. *J. Bacteriol.* **90**: 511-516. 1965.—Cytopathic effects (CPE) produced in HeLa cell cultures by two strains of mycoplasmas (F-11 and F-12) were studied by light and electron microscopy. CPE, which was marked by cytoplasmic vacuolization, did not appear to depend on intracellular mycoplasma infection. The cytopathogenic mycoplasmas appeared to be similar, both morphologically and in their intra- and extracellular distribution, to noncytopathogenic mycoplasmas previously studied by others. The probability that the CPE is related to depletion of essential nutrients is discussed, and the fine structure of the mycoplasmas is described.

Two strains of mycoplasma causing cytopathic effect (CPE) in tissue cultures and acidification of the medium have been discovered during attempts at isolation of viruses from human tumor tissues (Armstrong et al., 1965). The isolation of these agents, whether they have a direct association with the neoplasms in vivo or not, made it desirable to investigate these mycoplasmas in detail as to their growth in tissue cultures, their ultrastructure, and the characteristics of the CPE caused by them. Such studies employing light and electron microscopy are described in the present communication.

### MATERIALS AND METHODS

*Tissue culture.* HeLa cells (JJH line) were used in all experiments. They were grown in bottle cultures by use of Eagle's basal medium (BME) and 10% inactivated calf serum. After the cells formed a complete monolayer, they were maintained on BME and 2% inactivated calf serum. All cultures infected for morphological studies showed growth of mycoplasma colonies when subcultured on agar, whereas control cultures did not.

*Infection of tissue culture with mycoplasma.* The mycoplasma strains F-11 and F-12 were propagated in tube cultures of HeLa cells. After CPE was observed, the cultures were frozen and thawed twice. This material, which titered  $10^5$  TCID<sub>50</sub> per milliliter, was used for infecting Blake bottle cultures of HeLa cells.

The infecting material in amounts of 0.5 ml was added to 25.0 ml of maintenance medium. This

mixture was used to replace the maintenance medium of bottle cultures. After 3 to 4 days, the infectious medium was removed and replaced by maintenance medium. Marked CPE was evident after a total of 7 days. At this time the cells were harvested, fixed, and embedded for electron microscopy.

*Electron microscopy.* The cell sheets of infected bottle cultures were removed by incubation at 37 C with trypsin. The cells were pooled, sedimented by low-speed centrifugation, and the trypsin was discarded. The cell pellet was resuspended in 2.0 ml of phosphate-buffered saline (PBS), drawn into a syringe, and squirted into an equal volume of buffered osmium tetroxide contained in a plastic centrifuge tube. The cells were then pelleted in the fixative for 10 min. The cell pellet was removed, after cutting of the tube, and transferred to 50% ethyl alcohol, where it was dissected into smaller fragments. The cells were then dehydrated and embedded in epoxy resin. The material was sectioned with a diamond knife on a Porter-Blum microtome, contrast-stained with uranyl acetate, and viewed in a Siemens Elmiskop I electron microscope at a magnification of 10,000 X.

*Light microscopy.* Cultures of JJH HeLa cells were grown in 60-mm Falcon plastic petri dishes with 5 ml of a cell suspension containing 100,000 cells per ml in BME with 2% inactivated calf serum. The cultures were incubated at 37 C under 5% carbon dioxide, and after 1 day of incubation were inoculated with F-11 or F-12 HeLa passages with use of 0.2 ml per plate. Control cultures were not inoculated. Medium was changed at 3 days, and CPE appeared in infected cultures by the 4th

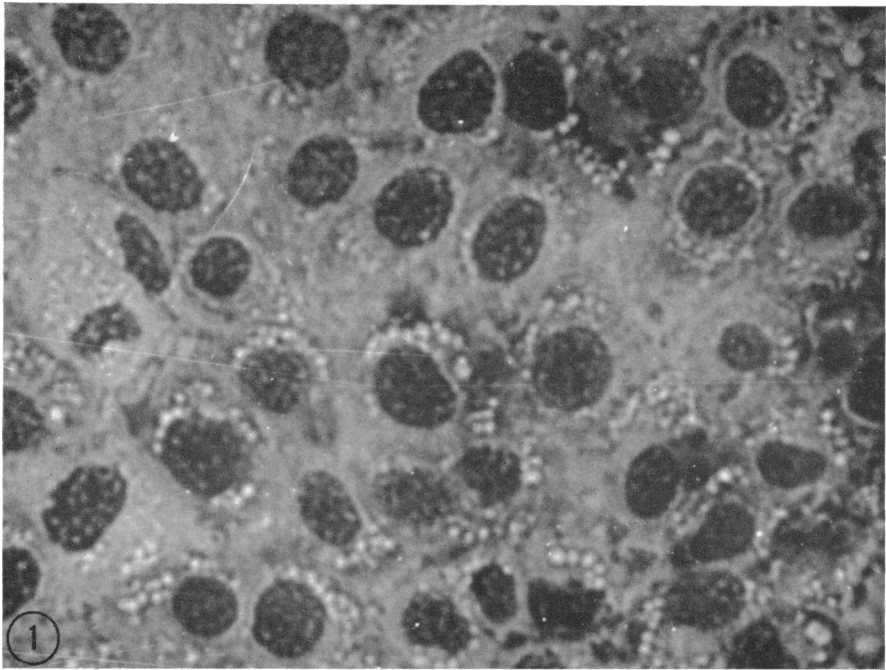


FIG. 1. *Cytopathic effect in HeLa cell culture caused by mycoplasma.  $\times 1,300$ .*

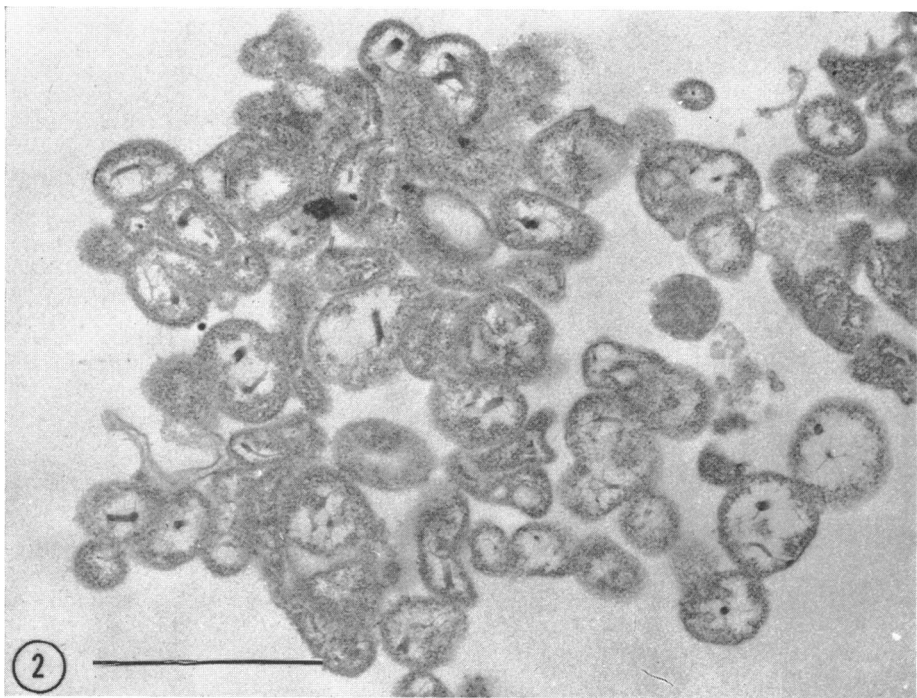


FIG. 2. *Extracellular aggregate of mycoplasma cells. The bar represents  $1 \mu$ .  $\times 30,000$ .*

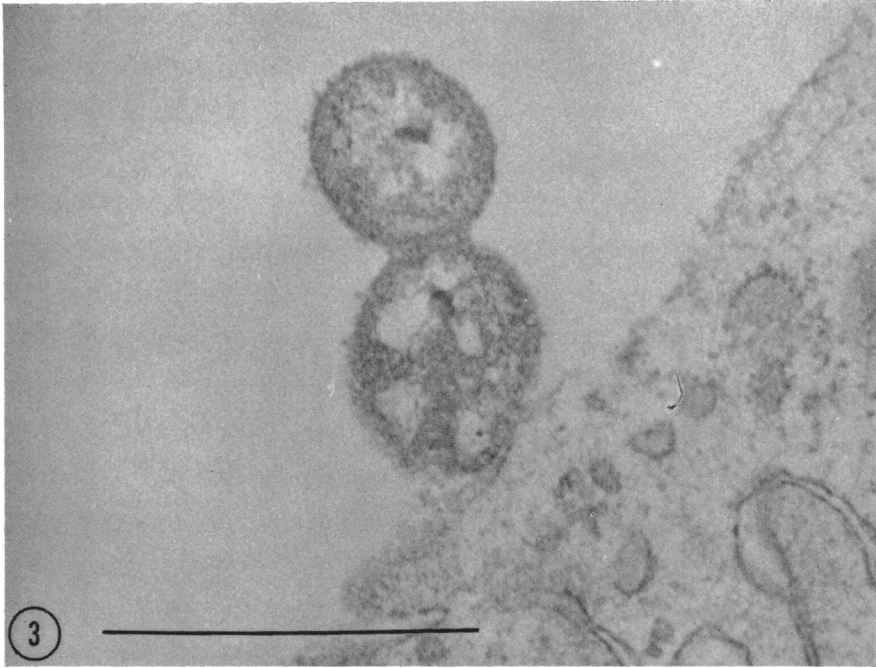


FIG. 3. *Mycoplasmas* attached to the plasmalemma of a *HeLa* cell. The bar represents  $1 \mu$ .  $\times 50,000$ .

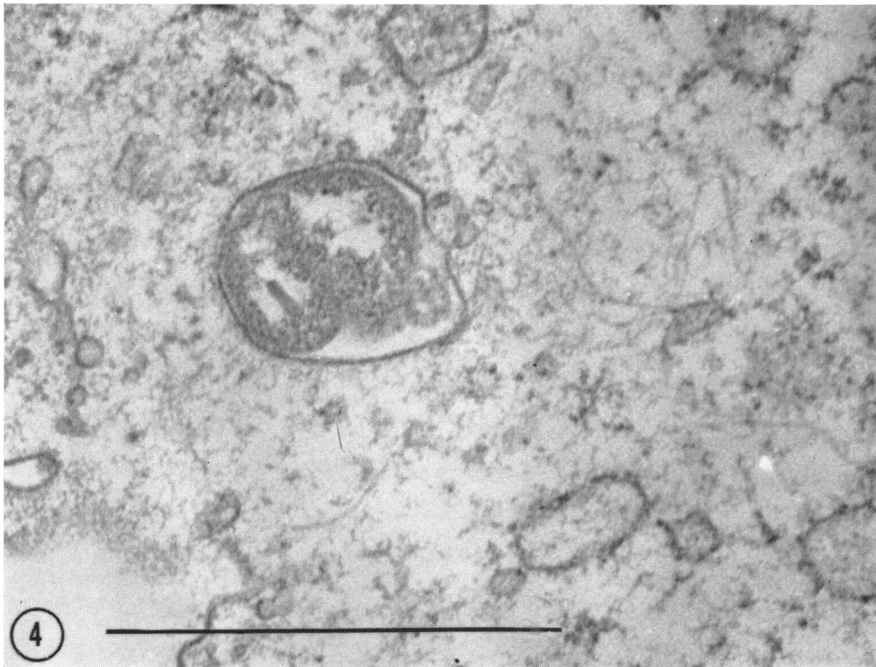


FIG. 4. *Mycoplasmas* in cytoplasmic vacuole of a *HeLa* cell. The bar represents  $1 \mu$ .  $\times 60,000$ .

day. Supernatant fluid was discarded on the 7th day after inoculation, the monolayers were washed, fixed with methanol for 10 min, and stained with May-Greenwald-Giemsa.

#### RESULTS

*Light microscopy.* Tubes inoculated with either F-11 or F-12 showed increased acidity by

the 3rd to 4th day, and by the 7th day patches could be seen where cells had detached from the glass. If previously untouched tubes were shaken at this time, a V-shaped streak with its apex toward the mouth appeared, because of cells detaching from the glass. Microscopically, the cells appeared gradually to lose their outer membrane, the cytoplasm became granular and then

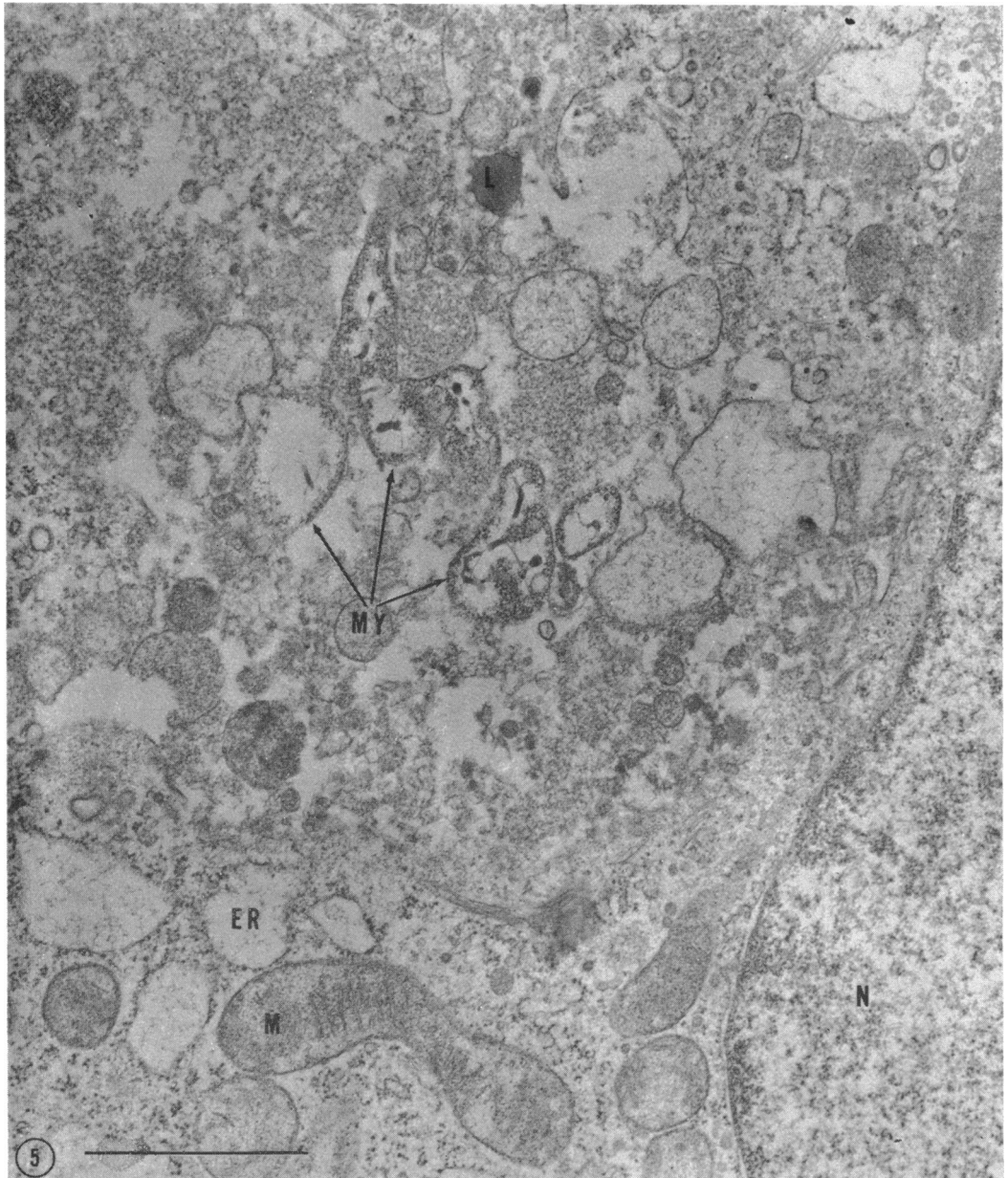


FIG. 5. *Mycoplasmas* free in cytoplasm of a HeLa cell surrounded by necrotic area. The bar represents  $1 \mu$ . Abbreviations: *My* = mycoplasmas; *M* = mitochondria; *L* = lipid bodies; *ER* = widened endoplasmic reticulum.  $\times 40,000$ .

vacuolated, and they finally disintegrated. Granulation occurred at the nuclear membrane and cell border, and shiny refractile vacuoles appeared in the cytoplasm. In heavy infections, refractile bodies the size of the vacuoles could be seen floating in the medium. With May-Greenwald and Giemsa (Fig. 1), the vacuoles did not stain, but dark blue-staining aggregates could be seen among the disintegrating cells. There was a morphological uniformity to the blue-staining material which was not seen in control cells during nonspecific degeneration. These uniform aggregates, which were seen most commonly in areas where cells had progressed beyond vacuolization to disintegration, were considered aggregates of mycoplasma, and observations under the electron microscope (see below) tended to confirm this suggestion.

*Electron microscopy.* The mycoplasmas were found in four locations relative to the parasitized cell. (i) Large aggregates were apparent extracellularly, sometimes in proximity to cells which did not show any morphological alteration. These aggregates often contained up to 50 particles. The diameter of some of these approached 5 to 6  $\mu$ , and thus it seems likely that they are identical with the granules seen under the light microscope in infected cultures (Fig. 2). (ii) Mycoplasmas were found attached to and fused

with the plasmalemma of the cells (Fig. 3). (iii) Single or groups of organisms were found in intracytoplasmic vacuoles of HeLa cells which otherwise showed little or no structural damage (Fig. 4). (iv) Mycoplasma particles found free in the cytoplasm of cells were always surrounded by marked necrosis of the surrounding cytoplasm (Fig. 5).

The organisms, whether extra- or intracellular, were uniform as to their basic structure, although they demonstrated a certain degree of pleomorphism due, in all likelihood, to their flexible limiting membrane. Depending on the plane of sectioning, densely stained nucleoids could be seen connected with each other and the surrounding cytoplasm of the agents by fine filaments. Dense granules, frequently lining the limiting membrane in a thick layer, were demonstrated in the great majority of the agents. The mycoplasma appeared round to ovoid in shape with a diameter from 200 to 500 m $\mu$ .

The pronounced CPE exerted by these agents manifested itself ultrastructurally by widening of the endoplasmic reticulum, resulting in vacuolization. Mitochondria were found to be distorted, and necrotic areas became apparent in the cytoplasm. These features were frequently evident in cells in the absence of detectable mycoplasmas. Nuclei of these cells did not seem

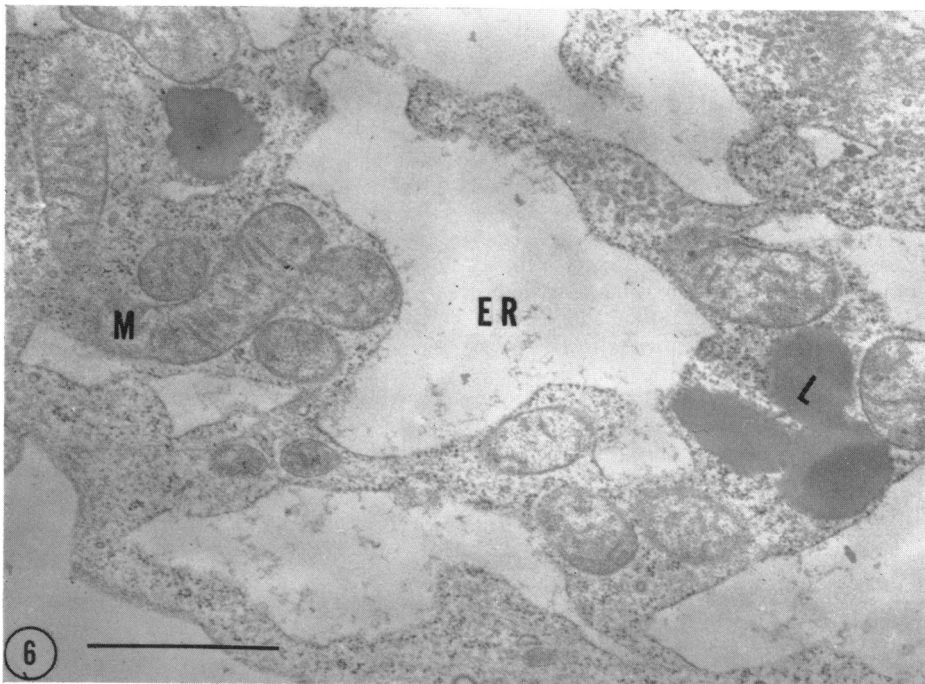


FIG. 6. Vacuolization of a HeLa cell. The bar represents 1  $\mu$ . Abbreviations are the same as in Fig. 5.  $\times 25,000$ .

to show any changes prior to complete disruption. Most of the described features can be seen in Fig. 6.

#### DISCUSSION

The isolate F-11 from the hemangioma is a mixture of a mycoplasma serologically related to *M. hominis* type 1 and a second mycoplasma related to the F-12 isolate. Mycoplasma F-12, from the fibroma, serologically belongs to a new group of mycoplasmas which cause extensive CPE in tissue cultures (Butler and Leach, 1964; Girardi et al., *in preparation*). Investigation of the infectious process by means of the electron microscope revealed a close similarity of the F-11 and F-12 mycoplasmas with regard to both their morphology and their action upon host cells. The results are similar to those reported previously by Edwards and Fogh (1960), who studied the structure of two different mycoplasma strains and their effect on human amnion cells. Of particular interest is the fact that one unidentified strain of mycoplasma caused a spontaneous latent infection of amnion cells, but electron microscopically the various types of cellular responses to the infection were identical with those described in this paper. Thus, the pronounced CPE caused by the isolates F-11 and F-12 from human tumor tissues seems to be a matter of degree of activity rather than of specificity. That is borne out also by the fact that F-11 and F-12 mycoplasmas themselves do not differ basically in their fine structure from others previously described.

The depletion of tissue culture medium of arginine by mycoplasmas has been described (Rouse, Bonifas, and Schlesinger, 1963; Pollock, Treadwell, and Kenny, 1963; Kraemer, 1964) and, in the first publication in this series, we showed that addition of arginine slightly altered CPE by both isolates as determined by light microscopy (Armstrong et al., 1965). Thus, the CPE of these strains, as compared with the ones that grow in tissue cultures without exerting such reaction, seems to be caused by either more rapid multiplication in this environment, or by a greater demand on nutrients present in the cultures. This appears reasonable in the light of the morphological evidence presented. Many tissue culture cells are in various stages of structural breakdown without any mycoplasma in evidence. Furthermore, it is unlikely that the ex-

tremely large aggregates of these organisms found extracellularly are solely derived from intracellular growth cycles. It is suggested that the mycoplasma can become attached to the plasmalemma of the cell and can enter the cell by pinocytosis. They may divide in cytoplasmic vacuoles or enter the cytoplasm proper by unknown pathways and multiply there, exerting a necrotic effect on the host cell. Yet the interior of the cells seems to be neither the only nor the main site of multiplication. It is likely that the mycoplasmas do multiply in the extracellular environment, and, by depleting the medium of vital metabolites, cause a breakdown of the host cell, therefore becoming manifest as CPE in the infected cultures.

#### ACKNOWLEDGMENTS

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