Mycoplasmas and Cell Cultures

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INTRODUCTION

Since the first report of mycoplasmal contamination in cell cultures (152), it has become apparent that a large number of cell lines used in biological studies throughout the world are contaminated with these microorganisms. To many investigators, mycoplasmas are regarded as a nuisance and have been ignored in experimental procedures, since the mycoplasmas often exert no obvious effect on the well being of the cell. In recent years it has become increasingly evident that mycoplasmas do indeed alter the cell in terms of macromolecular synthesis, stability of genetic material, and other parameters, including sensitivity to viruses and pharmacological drugs. It is the purpose of this review to collate present knowledge concerning the interrelationships between mycoplasmas and cells in culture and also the detection and eradication of these microorganisms.

It is not, however, the purpose of this review to consider extensively the structure and physiology of mycoplasmas. Mention will be made only of those properties that relate to mycoplasma-host cell interrelationships. The reader is referred to the excellent reviews published recently on the structure and function (144) and physiology of mycoplasmas (178, 153) for more extensive coverage.

CLASSIFICATION OF MYCOPLASMAS

For many years mycoplasmas were referred to as pleuropneumonia-like organisms (PPLO). Such terms are now in disfavor and the correct generic name, Mycoplasma, should be used. Recently the mycoplasmas have been classified into two separate families, the sterol-requiring Mycoplasmataceae and the sterol-nonrequiring Acholeplasmataceae (44). To the present time, over 30 named species of mycoplasmas have been included within the two families. In addition there are several unidentifiable isolates (L. Hayflick, personal communication). The above classification refers to classic (large-colony) mycoplasmas. It does not include T-strain mycoplasmas (so called, because they form tiny colonies approximately one-fourth the size of classic mycoplasma colonies) on agar. T-strain mycoplasmas were first isolated by Shepard from human urino-genital tracts (170). They have now been isolated from various animals. The T-strain group, as a whole, is unclassified at the present time. A unique requirement for urea (171), in addition to sterol requirement, for growth of these mycoplasmas has prompted the suggestion that they should be included as a separate genus in the order Mycoplastales (156a).

MORPHOLOGY OF MYCOPLASMAS

The mycoplasmal organism is distinctive for its small size of 130 to 300 nm and for the lack of a cell wall, the limiting boundary being a plasma
membrane similar in composition to that of an animal cell. The cells are highly pleomorphic, ranging from coccoid to filamentous, with a pronounced tendency of the filaments to form branching structures. Mycoplasma colonies are small, with diameters ranging from 100 μm to 1 mm. They usually have a characteristic “fried egg” appearance on agar, due to a tendency of the central core to grow down into the agar. The colony size of T-strain mycoplasmas is much smaller than that of the so-called classic mycoplasmas, ranging from 15 to 40 μm in diameter. Because of their small size, the mycoplasmas are difficult to detect in cell cultures by conventional light microscopy, and unless they produce observable changes in the structure or metabolic activities of tissue cells in culture they may go undetected.

**METABOLIC ACTIVITIES OF MYCOPLASMAS**

The mycoplasmas are an extremely fastidious group of organisms that require a complex medium for growth. Medium of Hayflick’s formulation (74), in which the human pathogen *M. pneumoniae* was first isolated (28), consisted of beef heart infusion broth, horse serum, and yeast extract.

All mycoplasmas that have been studied have an absolute requirement for nucleic acid precursors that can be satisfied by the free bases in certain instances (143), nucleosides (143, 145), oligonucleotides (143), deoxyribonucleic acid (DNA), or ribonucleic acid (RNA) (143, 146, 147). Nucleases have been isolated from mycoplasmas (134, 148), and the enzyme activity in one study appeared to be that of an endonuclease (120). As will be seen in later sections, this requirement for nucleic acid precursors can have profound effects on the metabolism of mycoplasma-infected tissue cells.

The utilization of amino acids is another metabolic activity of certain mycoplasmas that has a profound effect upon infected cultures. It was first shown by Smith (179, 180) that mycoplasmas of unknown species utilized arginine and glutamine rapidly with the production of ornithine and proline, respectively. Subsequently, it was shown that the mycoplasmas degraded arginine via the arginine dihydrolyase pathway, unlike mammalian cells that have the enzyme arginase (14, 164-166). Classic mycoplasmas can be divided into two broad physiological groups, the nonfermentative and fermentative strains. The breakdown of arginine is an energy pathway for nonfermenting mycoplasma species, 1 mole of arginine resulting in 1 mole of adenosine triphosphate (167). It should be noted, however, that the majority of fermenting mycoplasma species do not possess this enzyme pathway. The fermenting mycoplasmas obtain energy from the fermentation of various sugars via the glycolytic pathway. It is pertinent to emphasize the tremendous heterogeneity of mycoplasma species, especially in terms of physiological activity. This very heterogeneity leads to the broad range of effects on cell cultures that will be considered in most sections of this review.

**BIOCHEMICAL CHANGES IN HOST-CELL METABOLISM**

Powelson was the first person to show that arginine was depleted in contaminated cultures (139). Schimke et al. (167) noted that nonfermenting mycoplasmas utilize large amounts of arginine as a major energy source. In fact, 14% of the soluble protein from cell extracts of *M. hominis* consisted of enzymes of the arginine dihydrolyase pathway. Of the arginine utilized, 90% could be accounted for by the formation of ornithine, an amino acid that cannot substitute for arginine in mammalian cell cultures (41).

Powelson also showed that levels of glutamine in mycoplasma-contaminated cell cultures were below those of uninfected cultures, but not completely depleted (139). However, the level of glutamine in mycoplasma-infected cultures could be reduced from 2 to 0.2 mM without causing detectable changes in cell growth (91).

Depletion of essential amino acids has profound effects on cell metabolism. Eagle showed that mammalian cell cultures have an essential requirement for 13 amino acids (41), including arginine and glutamine. Certain of the nonessential amino acids derive their carbon skeleton from glutamine. Removal of any essential amino acid leads to cell death. The effect is irreversible if not rescued within a short time after depletion. Freed and Schatz (56) demonstrated that rescue of depleted Chinese hamster cells with the appropriate essential amino acid caused extensive chromosomal damage during the semisynchronous round of cell division (56). Such a “rescue effect” might take place in a mycoplasma-contaminated culture depleted of arginine when fresh medium is added at time of subcultivation or refeeding.

As mentioned in a previous section, it is mainly nonfermenting mycoplasmas that utilize arginine as an energy source. It is pertinent, therefore, to ask whether mycoplasmas deplete culture media of other essential amino acids, especially since it is known from studies of their nutrition in artificial medium that they require the presence of amino acids other than arginine for growth (179, 180). The few studies that have been performed to date...
would seem to indicate that no amino acid other than arginine is depleted below a minimal level necessary for survival of cell cultures. Chao et al. (29) studied the intracellular free amino acid pools of infected and control hamster fibroblasts and found that the ninhydrin-positive fractions were similar in all the lines tested with the exception of a depletion of arginine and an increase in citrulline and ornithine in the infected cultures. Stanbridge et al. (187) studied the growth of fermenting and nonfermenting mycoplasmas in cell-free Eagle's basal medium supplemented with nucleic acid precursors and dialyzed calf serum and found that the only amino acid which disappeared from the medium was arginine (in the nonfermenting cultures only). In certain instances, there was an increase in free amino acids in the medium which may have been due to a proteolytic action on the serum (Table 1). Only a few species of Mycoplasma have been studied in this system. It is entirely possible that strains requiring large amounts of other specific amino acids exist.

From a biochemical standpoint, perhaps the most interesting effects observable in mycoplasma-infected cells are the alterations in nucleic acid metabolism. Hakala et al. (67) showed that when HeLa cells are grown in a modified medium containing amethopterin they can utilize thymidine, thymidylic acid, and 5-methyl-cytosine deoxyribonucleoside as sources of DNA thymine. When the medium contained 5-bromodeoxyuridine (5-B UdR) in place of thymidine, growth also occurred. One strain of HeLa cells, subsequently found to be contaminated with mycoplasmas, did not grow in the amethopterin medium containing the deoxyribonucleosides. In addition the cells were resistant to inhibitory activity of 5-fluorodeoxyuridine (5-F UdR). Incubation of thymidine with this cell line, designated HeLa/PPLO, resulted in the cleavage of thymidine to thymine. No such cleavage was found with control cells. Cleavage of 5-F UdR to the free base 5-fluorouracil (5-FU) was noted, because the HeLa/PPLO cell line was resistant to 5-F UdR and 5-FU whereas a control line was 43 times more sensitive to the nucleoside 5-F UdR than the free base 5-FU. Other strains of mycoplasma were shown (67) to cleave thymidine, and evidence from growth studies suggested that 5-methyl cytosine deoxyribonucleoside, 5-bromodeoxyuridine, and 5-iododeoxyuridine were also cleaved. This substrate specificity led them to suggest that the

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*My coplasma* species were incubated in Eagle's Basal Medium supplemented with dialyzed fetal calf serum (10% final concentration) and nucleic acid precursors (20 mg% final concentration). Samples were incubated for a period of 13 days at 37 C. Free amino-acid concentrations are calculated as μmoles/0.5 ml. From E. Stanbridge, et al. (187).
mycoplasmas possessed a pyrimidine nucleoside phosphorylase similar to that isolated from horse liver (57).

Subsequent studies confirmed the nucleoside phosphorylase activity associated with the contaminated HeLa cells (79). The growth-inhibitory effect of the folic acid antagonist methotrexate, in mycoplasma-free HeLa cells, was prevented by simultaneous incorporation into the medium of glycine, hypoxanthine, and thymidine. The contaminated HeLa cells did not grow in this medium because of the cleavage of thymidine to thymine by the mycoplasmas. When 5-allyl-2′ deoxyuridine (5-AUdR), a compound that specifically inhibits nucleoside phosphorylase activity, was added, growth occurred. Added alone this did not support cell growth, but if 5-AUdR and thymidine were present together growth occurred, because the 5-AUdR inhibited the enhanced mycoplasma nucleoside phosphorylase activity. Sensitivity to 5-FUdR was also restored in the presence of 5-AUdR, again due to inhibition of the nucleoside phosphorylolytic activity that cleaved 5-FUdR to 5-FU. It is obvious from these findings that investigators screening for nucleic acid precursor analogues as potential chemotherapeutic agents in cell cultures must take mycoplasma contamination into serious consideration. Such a case in point is that studied by Neufahr et al. (121), who showed that the inhibition of vaccinia virus synthesis by 5-iododeoxyuridine was neutralized by the presence of M. arthritidis.

Inhibition of uptake of tritiated thymidine and uridine by mycoplasma-infected L cells was shown by Nardone et al. (118). Even when 1.0 μCi/ml was used, there was no incorporation of tritiated thymidine into nuclear DNA. However, the L cells continued to divide, and Nardone et al. attributed this to de novo synthesis of nucleic acid precursors.

Lability of host cell DNA in growing cultures of infected HeLa cells was observed by Randall et al. (141), who measured release of acid-soluble radioactivity into the medium. They had shown earlier the degradation of host cell DNA of HeLa cells infected with equine abortion virus (142). Subsequently, this was found to be due to a mycoplasmal contaminant that exhibited high deoxyribonuclease activity in the L cells (190).

Infection of BHK-21 cells with various mycoplasma species resulted in elevated levels of thymidine kinase, deoxyribonuclease, and ribonuclease (160). Levine et al. (102), studying patterns of RNA synthesis in serially propagated human diploid cells, found that with increasing passage level of the cultures new RNA species were observed that sedimented at 14 to 20S. There was also decreased uridine incorporation into 28S ribosomal RNA. Decreased incorporation of 14C-uridine into ribosomal precursor RNA was reflected in a decreased ribosomal RNA content per cell. Attempts were made to isolate mycoplasmas, but at this time such tests were negative. However, repeated attempts proved positive and in subsequent studies, with deliberately infected human diploid cells, Levine et al. (101) showed that the new RNA species were in fact mycoplasmal in origin. Synthesis of ribosomal RNA was almost abolished in contact-inhibited control and infected cultures. In contrast, incorporation of 14C-uridine into 14 to 20S was unaffected by cellular contact. Tritiated thymidine was incorporated into cytoplasmic “light” DNA, with a high turnover rate. The “light” DNA species was not characterized but was probably mycoplasmal DNA. Both fermenting and nonfermenting mycoplasma species caused these changes.

The presence of mycoplasmal RNA species in contaminated cell cultures has been confirmed by other investigators using a number of different cell systems. Markov et al. (111) found two new peaks at 16 and 23S, in addition to RNA profiles typical of total RNA of mammalian cells. Although these peaks constituted only a small proportion of the total cell RNA, they had a high incorporation rate of 32P label. Isolation attempts and electron microscope studies confirmed the presence of mycoplasmas. An interesting finding by Harley et al. (70) that may be useful in the detection of mycoplasmas is that the mycoplasmal RNA species in contaminated HeLa cells did not incorporate label in the presence of 14C-methionine (labeled in the methyl position), whereas the mammalian RNA did. The reason for this is that mammalian RNA is methylated by transfer of the methyl group from methionine, whereas mycoplasmal RNA is not (68).

It cannot be over emphasized in this review that all studies involving cell cultures should be stringently controlled for the presence of mycoplasmas. The alterations in metabolic activity of contaminated cells reviewed in this section should be some indication of the pitfalls that are present for the unwary or unconcerned biochemist.

**MYCOPLASMA EFFECTS ON CELL GROWTH AND MORPHOLOGY**

**Morphology**

The effects of mycoplasmas on the morphology of contaminated cell lines range from zero (26, 135, 155, 156) to increase in cytoplasmic granularity and retardation of growth (91, 185, 186) to frank cytopathology (3, 9, 24, 27, 54, 61, 66, 76, 84, 88, 137, 185, 186). The mycoplasmas may have such a drastic effect upon the cell cultures that the
whole cell population lyses (92, 94). Morphological "transformation" of hamster kidney cells mediated by mycoplasmas has been reported (107). The "transformed" cells exhibited a higher degree of malignancy than that of the parent type. It was subsequently shown that the "transformed" cells were probably selected from a preexisting population and were more mycoplasma resistant (161).

The effects of mycoplasmas on the morphology of cells in cultures vary according to the species of mycoplasma, the cell type, and the environment of the cell culture. The same cell-culture population infected with different mycoplasma species has given results varying from no effect to an overt cytopathic effect (CPE) (27). Kraemer et al. (94) found that infection of the mouse lymphoma cell line L5178 Y with certain mycoplasmas resulted in complete lysis of the culture. Other mycoplasmas produced no such effect. The lytic mycoplasmas had no cytopathic effect on other cell populations such as monkey kidney, hamster, or HeLa. The lytic factor was found to be a diffusible "toxin" that degraded arginine. Addition of excess arginine completely reversed the lytic effect (92). These lytic mycoplasmas were identified as _M. hominis_, a species known to possess the arginine dihydrolase pathway (167). Addition of excess arginine in other cell systems has also reversed the CPE (91, 137). Conversely, the inability of excess arginine to reverse CPE has also been reported (3, 9, 54). This is hardly surprising as it has been shown that _M. hyorhinis_, a mycoplasma that does not degrade arginine (14, 187), can induce CPE in cell cultures (24, 61, 84, 185, 186). It has been postulated that this mycoplasma and other fermenting strains exert their effect by a competitive utilization of nucleic acid precursors (187).

Characteristic morphological changes in the nuclei are often evident in mycoplasma-infected cultures. Cells undergoing degeneration show pyknotic nuclei, margination of chromatin, and nucleolar segregation (87, 185, 186). In addition, nuclei with areas of condensed chromatin have been seen in infected human diploid fibroblasts. Because of the characteristic morphology of these cells, they were described as "leopard cells" (61, 185, 186). The characteristic nuclei were shown to be similar in appearance (185, 186) to those induced by treating such cells with arabinosyl cytosine, an analogue of deoxycytidine and cytidine which interferes with DNA synthesis (77). The leopard-cell effect was also seen in chick embryo cells infected with _Acholeplasma laidlawii_ (88). Eradication of the mycoplasmas reversed the leopard-cell effect (185, 186).

Another interesting phenomenon was the segregation of nucleolar components into large, aggregated masses. Addition of arginine did not reverse or prevent nucleolar segregation, but eradication of the mycoplasmas did (87). Various mechanisms have been invoked to explain the segregation of nucleolar components produced by chemical, physical, and viral agents. These include inhibition of DNA synthesis and alteration of RNA and protein metabolism. The mechanism whereby mycoplasmas induce such changes is unknown.

The morphological changes cited above are associated with infection of cells by classic mycoplasma. Several years ago, Shepard infected HeLa cells with T-strain mycoplasmas and noted a progressive CPE in the infected cells. This observation has recently been confirmed in other cell systems, the degree of CPE varying with the type of cell culture used (113). Although naturally occurring contamination of cell cultures by T-strain mycoplasmas has not been observed, it is entirely possible that such contaminants will be found since these mycoplasmas are more ubiquitous than was originally thought.

**Growth**

Several investigators have demonstrated that the growth rate of cells infected with mycoplasmas is lower than that of control cultures (91, 185, 186). Adverse effects on the growth of tumor cell lines in suspension cultures have also been reported (106, 116). MacPherson and Allner (106) noted that mycoplasma-infected HeLa cells grew as monolayers but not in suspension. Growth in suspension was possible when the mycoplasmas were eradicated. Moore et al. (116) confirmed this finding with a number of human tumor cell lines. However, Earle's L strain of mouse fibroblasts was capable of growing in suspension culture even when heavily contaminated (21).

Recently, investigators showed that lymphocyte cultures in vitro could not be stimulated to divide when exposed to phytohemagglutinin (PHA), a potent mitogen, in the presence of nonviable suspensions of _M. hominis_, a nonfermenting mycoplasma (35). The lymphocytes were not killed since the inhibition could be reversed by washing the cells. Further studies characterized the inhibitor as protein in nature, fairly heat stable, and susceptible to neutralization by specific antiserum. Spitler et al. (184), also using nonviable mycoplasmas, showed that inhibition division was not due to a combination of the organisms with PHA or to competitive binding on the lymphocyte surface membrane. Other
investigators showed that only extracts of non-fermenting mycoplasmas inhibited lymphocyte division and that the inhibition was reversed by addition of excess arginine (15). In a series of elegant experiments, Simberkoff et al. (172) proved conclusively that the inhibition was due to the enzymes of the arginine dihydrolase pathway, which could be blocked by antiserum to these proteins or by addition of excess arginine. In addition, it was shown that antisera to a number of different nonfermenting mycoplasma species cross-reacted strongly in neutralization tests, a result suggestive of interspecies antigenic homogeneity of the enzymes.

There has been one report of stimulation of lymphocyte transformation in response to a fermenting mycoplasma, M. pneumoniae (103). Lymphocytes sensitized to M. pneumoniae in vivo were stimulated in vitro upon reexposure to the mycoplasmal antigen. An interesting finding was erythrophagocytosis by approximately 1% of the transformed lymphocytes. Erythrophagocytosis was not seen in cultures of normal lymphocytes stimulated by PHA.

KARYOLOGIC CHANGES IN MYCOPLASMA-INFECTED TISSUE CULTURE CELLS

Several investigators have demonstrated that mycoplasmas induce chromosomal aberrations in cultured cells (10, 50, 130, 131, 185, 186). Most commonly, the aberrations consist of achromatic gaps and chromatid breaks, but translocations, dicentrics, pulverization, and other gross abnormalities have been observed (Fig. 1 and 2). Fogh et al. (50) noted that chronic infection of the human amnion cell line (FL) with mycoplasmas led to a gradual reduction of chromosome numbers and to the appearance of three new chromosome varieties which were morphologically distinct from chromosomes seen in uninfected control cultures. The new varieties persisted after mycoplasmas had been eliminated from the culture. Although the appearance of the new varieties could possibly be indicative of mycoplasma-induced genetic changes, as suggested by the authors, it may equally well be due to selection of a preexisting population, especially since the FL line is heteroploid in nature and the

Fig. 1. Metaphase spread of chromosomes from an uninfected culture of human diploid cell strain WI-38. \( \times 1,500 \).
The induction of chromosomal abnormalities in human diploid cells in vitro has led to theories concerning the possible role of mycoplasmas in Down's syndrome (4), spontaneous abortion (96), and transformation (107). However, controlled studies of chronic mycoplasma infection of the human fibroblast cell strain WI-38, which has a stable diploid karyotype and a limited in vitro lifetime (73), have shown that all species of mycoplasma tested, both fermenting and non-fermenting, induced chromosomal aberrations (185, 186) but did not transform the cell population. Although the chromosome-breakage frequency remained high, no population with altered karyotypic characteristics arose. When the mycoplasmas were eliminated by treatment with aureomycin, the chromosome-breakage frequency dropped to that of the control level and the surviving cells retained their diploid karyotype. Both infected and treated cultures senesced in culture as did uninfected controls; that is, there was no extension of life span of the culture that might have heralded a transformation.

Aula and Nichols (10) found that the induction of chromosomal aberrations and mitotic inhibition in human leukocyte cultures infected with mycoplasmas appeared to be due to arginine deficiency and could be reversed by the addition of arginine. Support for this theory was provided by the studies of Freed et al. (56), who showed that Chinese hamster cells deprived of specific essential amino acids, including arginine, were mitotically inhibited, and that when rescued by addition of the specific amino acid in question, they underwent a burst of semisynchronous division accompanied by extensive chromosomal damage. Freed et al. suggested that protein synthesis in cells starved of arginine, due to mycoplasma infection or deliberate deprivation, was inhibited due to a depletion of the intracellular arginine pool. Interference with the incorporation of thymidine into DNA occurred after depletion of the arginine pool. The authors suggested that the inhibition of protein synthesis in the arginine-deprived cells led to a comparable interruption in chromosome replication. However, only mycoplasmas containing the arginine dihydrolase pathway were used in the studies of Aula and Nichols (10). Fermenting mycoplasmas which do not possess this pathway and do not
deplete the culture medium of arginine (187) also induce chromosomal aberrations (185, 186). The mycoplasma-induced chromosomal breakage, like that of viral-induced chromosomal damage, is very similar to breakage induced by inhibitors of DNA synthesis (123). As described in a previous section, all mycoplasmas that have been studied, both fermenting and nonfermenting, require nucleic acid precursors for growth (143, 145, 187). This requirement and the similarity in morphological and karyological changes in cells induced by inhibitors of DNA synthesis have led Stanbridge et al. (187) to hypothesize that the chromosomal damage is due to interference with cellular DNA synthesis, either by a competitive utilization of the intracellular pool of nucleic acid precursors or by actual degradation of host cell DNA by nucleases. Randall et al. (141) demonstrated that host cell DNA of mycoplasma-infected HeLa cells became unstable and that acid-soluble oligonucleotides were released into the medium. Paton and Allison (129) showed that the human diploid cell strain WI-38, used by Stanbridge et al. in their studies, when treated with exogenous deoxyribonucleases, exhibited a higher chromosome-breakage frequency than control cultures. The deoxyribonucleases tested included an endonuclease. Endonucleases have been isolated from mycoplasmas (120, 134), and mycoplasmal deoxyribonuclease has been isolated from mycoplasma-infected L cells (190).

Most recently, chromosomal aberrations have been induced in human lymphocytes infected with T-strain mycoplasmas (96). It will be interesting to see whether these mycoplasmas which lack an arginine dihydrolase pathway deplete the medium of some other essential amino acid or interfere with host cell DNA synthesis.

ASSOCIATION OF MYCOPLASMAS WITH TISSUE CULTURE CELLS

Although mycoplasma-host cell associations have been studied at the light-microscope level with stained preparations (51, 52, 76), it is difficult to assess whether the association is extra- or intracellular. Electron microscopic studies, however, indicate that the mycoplasmas reside predominantly in an extracellular environment bound to the cell membrane [(5, 6, 45, 52, 83, 84, 207, 208) Fig. 3]. The striking affinity of mycoplasmas for cell membranes may be due to the presence of specific receptor sites, as described later in this section. The mycoplasmas have also been observed in cytoplasmic vacuoles, free in

**Fig. 3.** Extracellular mycoplasmas attached to the plasmalemma of a HeLa cell. X 50,000. [From Hummeler et al. (84).]
the cytoplasm of necrotic or disintegrating HeLa cells (207), and in cells that have localized necrosis in the areas adjacent to the organism [(84) Fig. 4 and 5].

An interesting phenomenon associated with mycoplasma infection of cells is the development of long cytoplasmic processes (207) and microvilli (52). It is possible that such processes may envelop the mycoplasmas bound to the membrane without actually phagocytosing the organism, thus protecting it from the action of antibiotics or specific antisera. Supporting data for such a possibility will be presented in the section relating to eradication of mycoplasmas.

Several investigators have shown that mycoplasmas did not grow in cell-free tissue culture medium. Mycoplasmas did not grow in fresh medium or medium conditioned by previous contact with cell cultures (26, 98, 137, 139, 187). However, supplementation of fresh medium with yeast extract or nucleic acid precursors allowed propagation of the mycoplasmas (46, 137, 185–187). It is apparent that the absolute requirement of the mycoplasmas for nucleic acid precursors in order to replicate was not met by concentrations present in the fresh culture medium or released from cells in culture. Stanbridge et al. (187) have postulated a necessary intimate association of the mycoplasmas with the host cells, either membrane-membrane association or an intracellular environment, in order to obtain the necessary nucleic acid precursors by some, as yet undetermined, mechanism. They showed that when medium containing mycoplasmas was removed from cell cultures, filtered to remove cellular debris, and incubated, there was little further propagation of the mycoplasmas and they died within a few days (188).

Quantitative studies tend to lend support to this hypothesis. Larin et al. (98) demonstrated that the ratio of numbers of membrane-bound to extracellular *M. pneumoniae* organisms, infecting WI-38 cells, was dependent on the input multiplicity of colony-forming units. With low multiplicities of infection, cell-associated mycoplasmas predominated and at the highest dilutions only cell-associated mycoplasmas could be detected (98). After incubation for a number of days, during which time the mycoplasmas multiplied (or if a high multiplicity of infection was used), the numbers of cell-associated and extracellular mycoplasmas were similar. The cell-free culture

![Fig. 4. Mycoplasmas located in a cytoplasmic vacuole of a human embryonic kidney cell. × 50,000. (From Hummeler, Armstrong (83).)](http://mmbr.asm.org/)
Mycoplasmas and Cell Cultures

Medium did not support the growth of the organisms. These results may possibly be explained by saturation of cell receptor sites on the WI-38 cell membranes or leakage of essential nutrients from the WI-38 cells due to an interaction between them and the cell-associated mycoplasmas that would allow growth of the extracellular organisms. The predominance of cell-associated mycoplasmas was also noted by Levine et al. (101), using a number of different mycoplasma species.

Recent studies have shown that the avidity of mycoplasmas for cell membranes may be explained by the presence of specific receptor sites. Taylor-Robinson and Manchee (192, 193) reported that various mycoplasma species adsorb to HeLa cells and spermatozoa of bovine and human origin. They suggested the receptor site may be sialic acid, a finding in agreement with the sialic acid receptor sites on red blood cells that bind mycoplasmas (60, 151). Sobeslavsky et al. (182), testing the adsorption of various mycoplasma species to a number of tracheal cultures and red blood cells from avian and mammalian...
species, confirmed these findings but also noted that other, as yet unidentified, receptor sites were present. Manchee et al. (108, 109) extended their earlier investigations to include a variety of cell types and mycoplasma species. Although the studies are still at the preliminary stage, it is apparent that different types of cells have different receptor sites and, similarly, the mycoplasmas have different binding sites, even within the same species (108). Those cells that do not have neuraminic acid receptor sites may have glycoprotein sites. The binding sites on the mycoplasma membranes were not affected by neuraminidase; thus, it is suggested that protein components may be involved (109).

**EFFECTS ON VIRAL SYNTHESIS IN MYCOPLASMA-INFECTED CELLS**

Because of the profound effects that mycoplasmas may have on the metabolic activity of cells in culture, it is hardly surprising that they have some effect on viral synthesis in such cells.

Mycoplasmas were first suspected of interfering with viral synthesis by Brownstein and Graham (21). During a study of the replication of mengo-virus in L cells, they found periodic decreases in plaque titer of the stock virus. Subsequently, mycoplasmas were isolated from the L cells. Single-burst experiments in contaminated and mycoplasma-free cultures showed that the number of plaque-forming units per cell was 5 to 57 in the contaminated cultures and reproducibly greater than 300 in the control cultures.

Kagan et al. (88, 89) showed that arbovirus replication was inhibited when grown in mycoplasma-contaminated cells. However, Rouse et al. (157, 158) noted that the titer of dengue type 2 virus (an arbovirus) was not affected when grown in mycoplasma-contaminated cells.

Rouse et al. (157, 158) found that the replication of adeno-viruses was dependent on the presence of arginine. Depletion of this amino acid by contaminating mycoplasmas resulted in a significantly lower number of plaques (10- to 100-fold) than did control cultures infected with adenoviruses but free of mycoplasmas. The inhibition of plaques could be reversed by adding excess arginine. Although not identified in these studies, it is probable that the contaminating mycoplasmas were nonfermenters. Subsequent studies have shown that the arginine-dependent step involved in adenovirus synthesis involves a maturation factor (159, 162). The factor involves a component of P antigen (an antigen within the virus capsid) that is required for assembly of the virus. Capsid antigens were still synthesized in the absence of arginine.

Arginine-dependent steps in the replication of cytomegalovirus (115), herpes simplex (17, 191), polyoma (200), simian virus 40 [(SV40) 62], vaccinia (174), measles (154), and poliovirus (1) have been documented. Of these viruses, inhibition of growth due to mycoplasma contamination has been demonstrated with measles (154) and vaccinia (174). Singer et al. (174) showed that the synthesis of vaccinia virus was inhibited by the nonfermenter *M. arginini* but was not affected by the fermenter *M. hyorhinis*, a clear indication again of the heterogeneous effects of mycoplasmas. Hargreaves and Leach however, studying the sensitivity of mycoplasma-infected HeLa cells for the growth of different viruses, found that the growth of vaccinia was in fact enhanced by the presence of fermenting and nonfermenting mycoplasmas (69, 100). Obviously, more studies are necessary to clarify the above results.

It will be interesting to see whether mycoplasmas affect other viruses that have an arginine-dependent step in their biosynthetic pathway.

Somerson et al. (183) found that the growth of Rous sarcoma virus (RSV) was inhibited by *M. orale* type 1 in a number of different cell cultures. Suppression of Rous-associated virus (RAV) was also evident, as measured by the absence of avian leukosis complement-fixing antigen in mycoplasma-infected chick embryo fibroblasts. Suppression of the formation of transformed foci was also noted, a finding confirmed by Ponten and MacPherson (138), who found complete inhibition of focus formation by RSV when the chick embryo fibroblasts had been previously infected with *M. hominis*. Presence of mycoplasmas has also been shown to be responsible for a prolonged time for the appearance of transformed foci of human amnion cells infected with SV40 (49).

Fowlpox virus (FP) grown in chick embryo fibroblasts contaminated with *M. gallisepticum* showed approximately a 1-log increase over control plaque-forming unit titers (59). In vivo determinations of FP titers were accomplished by inoculation of appropriate dilutions of FP onto the scalps of day-old chicks and by examination for pocks 7 days later. FP alone consistently gave titers approximately 1 log lower than the in vitro plaque count. FP-mycoplasma mixtures, however, were 5 to 6 logs lower than the plaque titer. Reconstruction experiments using fresh FP-mycoplasma mixtures showed no loss of infectivity. It was shown that at least three cycles of replication of FP in mycoplasma-contaminated cells were required before a significant decrease in the in vivo titer was apparent. This decrease in the in vivo titer was paralleled by a change in
the in vitro plaque morphology from large to small plaques in the mycoplasma-contaminated cell cultures. Such a finding has distinct importance, especially for virus vaccine production. There have been a number of reports of veterinary vaccines contaminated with mycoplasmas (18, 26, 66). Aside from the possibility that pathogenic mycoplasmas may be inoculated into the recipient along with the vaccine, there is the possibility that virulence or actual titer of the virus may be masked by the contaminating mycoplasmas. Such considerations have led the licensing authorities in many countries to include a quality-control test for the presence of mycoplasmas (F. T. Perkins, personal communication).

Inhibition of virus synthesis in many cases can be traced to a depletion of arginine. More puzzling are the reports of enhancement of virus titers (69, 100, 173, 175). As previously mentioned, Hargreaves and Leach (69) found that vaccinia and respiratory syncytial virus titers were enhanced in the presence of mycoplasmas. It has been demonstrated that rhinovirus RNA synthesis was greater in *M. pneumoniae*-infected KB cells than in mycoplasma-free KB cells (114). This stimulatory effect could also be produced by mycoplasma broth medium constituents, especially yeast extract (48) which contains an abundance of nucleic acid precursors. As previously stated, mycoplasmas are capable of degrading host-cell DNA and it is therefore possible that an increased level of such acid-soluble products could be made available for viral replication. Singer et al. (175) found an increase in yield of vesicular stomatitis virus (VSV) when a low multiplicity of infection was used in cells infected with *M. arginini*. No such enhancement was found with higher inputs of virus, a result indicating that several cycles of viral multiplication were necessary to achieve an amplification effect.

Subsequent investigations of the effect of mycoplasma infection on the production of interferon revealed no detectable levels in the supernatant fluids of cell cultures infected with mycoplasmas alone (173). Other studies on the influence of mycoplasma on the production of interferon have shown that mycoplasmas alone have little effect (8, 25, 205). Interferon levels induced by infection with Semliki Forest virus (SFV) were lower in mycoplasma-infected cells and yields of SFV were significantly increased. The mycoplasma-infected cells were also less sensitive to exogeneously supplied interferon. However, other investigators have found some suggestion of increased production of interferon when mycoplasma and virus are present together (25, 88, 205).

These conflicting results may again be an indication of the widely diverging properties of mycoplasmas. It will be necessary to study different combinations of mycoplasmas and cell populations to determine whether mycoplasmas do indeed induce the synthesis of significant levels of interferon.

**CASES OF MISTAKEN IDENTITY**

Mycoplasmas have a number of properties in common with viruses. Such criteria have led to erroneous conclusions that the agent being studied is a virus (201). Probably one of the most confusing properties is the capacity to produce CPE in cells. Other common properties are their filterability through 0.22-nm Millipore membrane filters (117), growth inhibition by specific antisera (43), sensitivity to chloroform and ether (74), hemagglutination (2), hemadsorption (19, 40), resistance to certain antibiotics, morphology by electron microscopy, and ability to induce chromosomal aberrations and morphological changes in infected cells.

One such case of mistaken identity that generated much interest at the time was the finding of agents in bone-marrow material from leukemic patients that caused CPE in tissue culture (119) and were identified by electron microscope observations as viruses (85). Subsequent studies showed that the "virus" was indeed a mycoplasma (65), later identified as *M. pulmonis* (47). Although unfortunate, this finding stimulated a search for a potential etiology of mycoplasmas in malignant disease. These organisms have now been isolated directly from a variety of malignant tissues and indirectly via tissue culture. The general consensus at this time is that the mycoplasmas are merely "passengers," as are bacteria and fungi which are often found in human leukemic tissue; such colonization is due to the impaired immune mechanisms of the leukemic individual (75).

A similar case was the finding that the A-1 agent, isolated from an intergenic plasma pool and originally thought to be a virus (127), was in fact *M. gallisepticum* (128). Most recently, an electron microscope study of rubella virus in cell cultures tentatively identified extracellular particles as rubella virions (30). Although isolation of mycoplasmas was attempted, the results were initially negative (T. S. L. Beswick, personal communication). However, the agent was subsequently identified as *M. orale* type 1 (38).

The above are examples of mistaken identity that were happily resolved. It can be left to the reader's imagination how many reports of "virus-like" particles may well be describing mycoplasmas.
DETECTION OF MYCOPLASMAS IN TISSUE CULTURE

The magnitude of mycoplasma contamination of tissue culture cells has become apparent in recent years, and many investigators have attempted to find more sensitive methods for detection of these organisms. In most cases, the test conceived will not detect all mycoplasmas, either because of their differing growth requirements or the inherent insensitivity of the test employed.

Possibly the simplest and most direct method is isolation of the mycoplasmas on solid medium. The methodology has been reviewed in detail by Hayflick (74) and MacPherson (105), and no further discussion will be made here. Although this method will detect the presence of most mycoplasma contaminants, there are reported instances of more sensitive tests. House and Waddell (82) showed that feeder layers of mouse embryo cells overlaid with agar were more sensitive than Hayflick’s medium alone. Zgoniak-Nowosielska et al. (206) found the presence of baby hamster kidney (BHK-21) cells necessary for the growth of certain mycoplasmas. The cells were grown in suspension in agarose medium and were infected with rabies and lymphochorio-meningitis virus. The virus pools were subsequently found to be contaminated with mycoplasmas. Both the viruses and mycoplasmas formed plaques, easily distinguishable from each other. The mycoplasmas could not be directly grown in mycoplasma broth or on mycoplasma agar, but they could be grown after a single passage on BHK-21-agarose medium. It would seem that the mycoplasmas had become adapted to a cellular environment and that a “weaning” period was necessary before growth could be established in cell-free medium.

Isolation of mycoplasmas on solid medium is often attempted with cell-culture fluid alone. Because of the avidity of mycoplasmas for the cell membrane, such attempts may give misleading, negative results (37, 98, 101, 185, 186). Cells should also be included in any sample tested. Although logic would dictate the homogenization of cells to release any intracellular mycoplasmas, normal tissue extracts from HeLa and L cells have been shown to possess mycoplasmicidal activity (90).

Several chemical and serological methods have also been proposed for the detection of mycoplasmas. A rapid chemical method utilizing a color test for the presence of citrulline and indication of arginine deiminase activity agreed completely with culture isolations (13). However, later studies showed that possession of this enzyme pathway is a property of nonfermenting mycoplasmas, and most fermenting mycoplasmas are negative in this test (14). The cleavage of thymidine has been proposed as a diagnostic test (81), but again strains have given negative results even when large numbers of organisms were present (82). Detection by use of fluorescent antibody has been used with success (12), but because it is becoming increasingly obvious that many different species of mycoplasmas commonly contaminate tissue cultures (75), multivalent antisera would be required. Also detection of low-level contamination would be difficult. Staining methods for direct demonstration of mycoplasmas, either extracellular or intracellular (51, 52, 169), also suffer from lack of sensitivity (37).

Perhaps one of the more promising approaches is the recognition of mycoplasmal RNA species in contaminated cells (70, 101, 111). The RNA profiles, 16 and 23S, are similar for all prokaryotes, and therefore not necessarily indicative of mycoplasma contamination alone (111). The mycoplasmal RNA species can be further distinguished from mammalian RNA by the absence of methylation (70). Labeling of mycoplasmas with radioactive uridine and thymidine and isopycnic banding of the whole particles in a 15 to 60% sucrose gradient at a density of 1.20 to 1.24 g/cc (194) has been demonstrated. Todaro et al. (194) demonstrated the presence of characteristic banding (later confirmed by electron microscopy to be mycoplasmas) in samples in which no mycoplasmas could be grown on solid medium. Quantitative tests of the sensitivity of this method are in progress.

It would appear that any one test is insufficient to detect all mycoplasma contaminants and that repeated attempts are necessary to detect low-level contamination. Although Hayflick’s statement that “the definition of the Order Mycoplasmatales presupposes a demonstration of characteristic growth on agar and any method circumventing this absolute requirement begs the question” (74) is essentially correct, in practical terms it is apparent that certain mycoplasmas have become so adapted to a cellular environment that growth is negligible on solid media available at the present time. Until more optimal media are available, such mycoplasmas may possibly be detected only by chemical or physical means. Tests that would be sensitive enough to detect low numbers of all types of these heterogeneous organisms are still to be discovered.

FREQUENCY OF MYCOPLASMA CONTAMINATION

Since the first report of cell cultures contaminated by pleuropneumonia-like organisms
[a term previously used to describe mycoplasmas (152)] there has been a multitude of such reports. Summaries of published results from various sources noted that up to 60% of the several hundred cell lines tested were contaminated (74, 105, 202). In one study of 60 cell lines, 86.7% were contaminated (126). In what is probably one of the most extensive studies performed, we have tested over 3,000 tissue culture samples, from many different laboratories, during the past 3 years and have isolated mycoplasmas from 12% of the samples (N. Pleibel and L. Hayflick, unpublished data). Primary cultures are only rarely contaminated (64, 78, 94, 106).

**SOURCES OF MYCOPLASMA CONTAMINATION AND THEIR POSSIBLE PREVENTION**

The source of mycoplasma contamination has been a perplexing problem. Until 5 or 6 years ago, it seemed that the majority of tissue-culture contaminants were of human origin (74) and, therefore, could originate from the faulty aseptic techniques of personnel carrying mycoplasmas in their oropharynxes. The contaminant, once established in a cell culture, could easily be transmitted to other cultures by aerosolization. O'Connell et al. (124, 125) demonstrated that aerosols were created in tissue bottles during trypsinization. When a burette was used to add medium to freshly trypsinized mycoplasma-infected cultures and then was used to add medium to mycoplasma-free cultures, 50 to 75% of them became contaminated. More recently, mycoplasmas of presumed avian (42, 75), swine (24, 61, 75), and murine (47) origin have been isolated. The saprophytic strain *M. laidlawii* (75), now reclassified as *Acholeplasma laidlawii* because of its nonrequirement for sterols for growth, and other sterol nonrequirers such as *A. granularum* (196) and as yet unclassified species (197, 198) are becoming increasingly common cell-culture contaminants. The nonhuman origin of these mycoplasmas raises questions as to the source of these contaminants, although the degree of host specificity of mycoplasmas is itself in question since swine mycoplasmas (75) and *A. laidlawii* (75, 110, 149) have been isolated directly from human clinical material. Constituents of cell-culture medium, such as serum and trypsin, have been frequently tested for the presence of mycoplasmas, with negative results. Within the past year, however, mycoplasmas have been isolated directly from commercial serum (M. F. Barile and G. Kern, personal communication) and also directly from the blood of calves and primary cell cultures of kidneys obtained from the same animals (L. Stipkovits, personal communication). Contamination of primary human embryo cultures may be effected by passage of the fetus through the birth canal, due to the high frequency of colonization of the urinary-genital tract by mycoplasmas. Mycoplasmas have been isolated directly from the organs of aborted human embryos (75). Vertical transmission of mycoplasmas in chick embryos has been well established; therefore, contaminated primary cultures of such tissue is possible.

Sabin has suggested that the mycoplasmas come in “on the wind” (163), a suggestion not without merit since there has been shown that mycoplasmas survive aerosolization to the same degree as bacteria when conditions of temperature and humidity are optimal (16, 95, 203). It is entirely possible that any one or all of the above suggestions are correct. Prevention of mycoplasma contamination is also a problem. Since the advent of antibiotics, the use of these compounds in cell cultures has encouraged inadequate aseptic technique. As will be seen in the section on eradication of mycoplasmas, antibiotic-resistant strains are becoming increasingly more common. Laboratories that have encountered this problem have found that the most successful method of prevention is the use of antibiotic-free media. The use of such media of necessity requires strict aseptic technique that should prevent contamination from the tissue culturist himself or from other infected cultures. Laminar-flow hoods are also successful in combating mycoplasma contamination by screening out airborne particles.

**ERADICATION OF MYCOPLASMAS**

It is becoming increasingly more difficult to eradicate mycoplasmas from contaminated cell cultures. Antibiotic treatment has been the system of choice for many investigators. Mycoplasmas have been eliminated by treatment with kanamycin (53, 91, 132, 133, 135), tetracyclines (80, 83, 99, 132, 133, 185, 186), tylosin (58, 132, 133, 189), erythromycin (176), and lincomycin (7) Tables 2-4. However, resistance to all these antibiotics has also been encountered (22, 37, 58, 71, 80, 133, 140, 189, 203). Such resistance can be easily induced by exposure of the organism to increasing concentrations of the antibiotic (133). Factors that influence the sensitivity of the mycoplasmas are manifold. Such factors include the strain of mycoplasma, the strain of cell culture, and the serum content of the medium (99). The effects of antibiotics on mycoplasmas themselves warrant a review. Excellent papers on this subject have been written by Newnham and Chu (122), Jao and Finland (86), and Perlman et al. (133). It has become increasingly obvious that the concentration of drug that will inhibit
TABLE 2. Sensitivity of strains of Mycoplasma pneumoniae, M. hominis, and M. fermentans to antibiotics and sodium aurothiomalate in vitroa.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Minimum inhibitory concn of drug (µg per ml) for</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M. pneumonia</td>
</tr>
<tr>
<td></td>
<td>Strain Hettet (FH)</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>&gt;250</td>
</tr>
<tr>
<td>Cephaloridine</td>
<td>100</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>1.5</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>&lt;4.0</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>6.25</td>
</tr>
<tr>
<td>Lincomycin</td>
<td>25</td>
</tr>
<tr>
<td>Methicillin</td>
<td>&gt;250</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>25</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>1.5</td>
</tr>
<tr>
<td>Sodium aurothiomalate</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

a From Stewart et al. (189).

b Mean of six tests.

c Not tested.

TABLE 3. Sensitivity of strains of Mycoplasma salivarium to antibiotics, sodium aurothiomalate, and tylosin tartratea.

<table>
<thead>
<tr>
<th>Drug</th>
<th>No. of strains tested</th>
<th>No. of strains with minimum inhibitory concn (µg/ml) of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.4</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>84</td>
<td>0</td>
</tr>
<tr>
<td>Cephaloridine</td>
<td>18</td>
<td>0</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>84</td>
<td>3</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>84</td>
<td>0</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>84</td>
<td>0</td>
</tr>
<tr>
<td>Lincomycin</td>
<td>33</td>
<td>2</td>
</tr>
<tr>
<td>Methicillin</td>
<td>84</td>
<td>0</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>84</td>
<td>0</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>84</td>
<td>3</td>
</tr>
<tr>
<td>Sodium aurothiomalate</td>
<td>84</td>
<td>2</td>
</tr>
<tr>
<td>Tylosin tartrate</td>
<td>42</td>
<td>3</td>
</tr>
</tbody>
</table>

a From Stewart et al. (189).

All strains insensitive to amoxicillin and methicillin at 250 µg/ml.

TABLE 4. Sensitivity of strains of Mycoplasma orale type I to antibiotics, sodium aurothiomalate, and tylosin tartrate in vitroa.

<table>
<thead>
<tr>
<th>Drug</th>
<th>No. of strains tested</th>
<th>No. of strains with minimum inhibitory concn (µg/ml) of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.4</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Cephaloridine</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Chloramphenicol</td>
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<td>0</td>
</tr>
<tr>
<td>Erythromycin</td>
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<td>0</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Lincomycin</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>Methicillin</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Sodium aurothiomalate</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Tylosin tartrate</td>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>

a From Stewart et al. (189).

All strains insensitive to amoxicillin and methicillin at 250 µg/ml.

mycoplasmas in vitro on agar and in broth often has no effect in a cell-culture system, and much higher concentrations are required for inhibition. Shedden and Cole (169) found that mycoplasmas in association with cell cultures were resistant to 500 µg of kanamycin per ml, whereas they were sensitive to 25 µg/ml in vitro. It is possible that the mycoplasmas, when attached to cell membranes, become encircled by cytoplasmic processes (52, 207), rendering them inaccessible to the drug. Hummeler and Armstrong (83) were unable to isolate mycoplasmas from cells treated with tetracyclines, but mycoplasma-like particles could still be seen by electron microscopy. Cross et al. (37) treated cells with aureomycin and found that intact cells and tissue-culture fluids were negative for mycoplasmas but that they could be isolated from disrupted cell pellets. Results of antibiotic therapy on patients suffering with atypical pneumonia caused by M. pneumoniae suggest a similar protective effect. Patients continued to secrete M. pneumoniae after treatment.
with tetracycline or erythromycin (55, 177), although the secretions contained concentrations of the antibiotics considerably in excess of those which inhibited the same mycoplasmas in vitro (177).

Other chemical compounds such as gold salts (39, 112, 168, 189), polyene antifungal agents (97, 122), cationic agents (36), and nonionic detergents (150) have also been used with success, but again resistance to these agents was also demonstrated.

It would appear that any single chemical agent is incapable of eradicating all strains of mycoplasmas from infected tissue cultures. Mixtures of antibiotics have been suggested as possible solutions (11, 22), but again the specter of multiple resistance is likely to arise. Hypotonic treatment of cells to increase their permeability to antibiotics was used successfully to eliminate mycoplasmas, but was also extremely harmful to the tissue-culture cells (64, 199). Such a treatment may well subject the cells to undesirable selection.

Other methods of decontamination have been equally unrewarding. Hayflick (72) reported that maintenance of contaminated HeLa and L cells at 41°C for 18 hr killed certain strains of mycoplasmas without irreversibly damaging the tissue-culture cells. Other investigators found the method unsatisfactory because the treatment damaged cells beyond recovery or failed to inactivate the mycoplasmas (78, 199).

Mycoplasmas have been eliminated from cell cultures by treatment with specific antiserum (78, 136, 199, 203). The specificity of this system is an obvious disadvantage, for identification of the contaminating mycoplasma must be established before the correct antiserum can be used. There has already been one report of two distinct species infecting the same cell culture (78). As mentioned previously, many serologically different strains of mycoplasma are now being isolated from tissue cultures, including species as yet unclassified (197, 198). Multivalent antisera would be required to eliminate such contaminants. Also, the antiserum treatment would have no effect on intracellularly residing mycoplasmas, and indeed may have little effect on extracellular mycoplasmas tightly enveloped by cytoplasmic processes (Fig. 6).

The eradication of mycoplasmas appears to be a difficult task, and although any one or a combination of the above methods may be successful, the adage "prevention is better than cure," so correctly stated by MacPherson (105), is certainly

**FIG. 6.** Mycoplasma (M) closely associated with the surface of a HeLa cell. Shown are cytoplasmic processes (CP) that envelop the mycoplasma. × 31,000. (From Barile, M. F., 1965, Wistar Institute Symp. Mono. No. 4, p. 171.)
applicable. Master seeds of cell cultures should be stored in liquid nitrogen or in a deep-freeze as early as possible, as suggested by MacPherson. Propagation in antibiotic-free medium from the outset is advisable.

**USE OF TISSUE CULTURE TO STUDY MYCOPLASMAS**

The bulk of this review has been concerned with the effects of mycoplasmas on cell cultures and how they can be eliminated. As a mycoplasmologist, I should also like to devote a section of this review to the applicability of tissue-culture systems to the study of mycoplasmas, especially those that are pathogenic. It may come as a surprise to those not involved in the study of mycoplasmas to know that they are important pathogens of poultry, cattle, swine, rodents, goats, and man.

Cell-culture systems have been used for many years in the study of viruses, in relation to virulence, but it is only very recently that mycoplasmas have been studied in this regard. The interesting finding that cells may bear specific receptor sites for mycoplasmas (60, 108, 109, 182, 192, 193) has led to in vitro studies of the human pathogen *M. pneumoniae* in various tissue-culture systems (32, 34, 104, 182). Tracheal organ cultures infected with virulent *M. pneumoniae* showed a more rapid loss of ciliary activity than did those infected with attenuated strains (34). Ciliary damage has also been demonstrated with virulent strains of *M. mycoides* var. *capri* (23, 31, 32) and *M. gallisepticum*. The damage caused by *M. mycoides* var. *capri* appears to be due to hydrogen peroxide production (31), a factor already associated with virulence in respiratory infections caused by *M. pneumoniae* (33, 104, 181).

Many pathogenic mycoplasmas are extremely fastidious. The human pathogen *M. pneumoniae* was grown in tissue culture for many years before a suitable synthetic medium became available for its propagation (28). The causal agent of enzootic pneumonia in pigs was also first isolated in tissue culture (20, 63).

**CONCLUSIONS**

It is apparent that mycoplasmas can have drastic effects on the metabolic activities of cells in culture. These effects may or may not include morphological and karyological changes in the infected cells. Biochemical and cytological results found in mycoplasma-contaminated cell cultures are therefore open to misinterpretation, as are similar studies of viruses, pharmacological and carcinogenic agents. Due to the heterogeneity of mycoplasmas one cannot predict the effects of such organisms upon infected cells, and for this reason any experimental results based on mycoplasma-contaminated cells should be considered suspect.

Detection and eradication of mycoplasmas is often difficult, and researchers should attempt to prevent contamination where possible by the use of antibiotic-free medium and sterile work areas.

**ACKNOWLEDGMENTS**

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