MYCOPLASMAS IN TISSUE CULTURE

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SUMMARY

Mycoplasmas are frequently found as contaminants in tissue-cultured cells. Infections may be inapparent or cause severe cytopathic changes. The source of primary contaminations in most cases is probably the upper respiratory tract of man. The wide dissemination of infections in cultures most probably occurs as a result of aerosols set up during the processing of contaminated cultures. Mycoplasmas in cultured cells may cause chromosome aberrations, degradation of the host cell DNA, and morphological transformations. They cleave thymidine and its related structural inhibitors and also degrade arginine. They inhibit the growth of adenovirus and Rous sarcoma virus and no doubt affect others. A number of antibiotics which are relatively non-toxic for cells in culture are active against mycoplasmas and may be used to cure infected cells. Mycoplasmas interfere with the biochemistry of the cell at many points and no one working with tissue cultures can afford to ignore them. Stringent aseptic techniques are the best safeguard against primary infections and cross-contaminations.

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INTRODUCTION

Mycoplasma contamination of tissue cultures has been recognized for almost 10 years, but it is only comparatively recently that an adequate understanding has been gained of the source of the contaminants and the reasons for their wide dissemination in cultures. There have also been many recent observations on the undesirable results of such contamination. The present review discusses the problem and possible methods of control.

FREQUENCY OF MYCOPLASMA CONTAMINATION OF TISSUE CULTURES

Since the first demonstration by Robinson, Wichelhausen & Roizman (1956) that a number of cultured cell lines were contaminated with mycoplasmas, there have been similar reports from many laboratories using cultured cells. Summarizing the published results from ten reports, 267 out of 454 (59%) of cultures examined were contaminated with mycoplasmas. Successful isolations probably give a conservative estimate of the proportion of cultures actually infected, since cultures with low levels of contamination may not always yield positive isolations on artificial media. Repeated attempts may be necessary to reveal contamination in some cultures (Macpherson & Allner, 1960; Pollock, Kenny & Syverton, 1960). Freshly trypsinized cells have a reduced mycoplasma content and the stage of growth of a culture will influence the yield of mycoplasmas. Kraemer, Defendi, Hayflick & Manson (1963) have shown that the conventional media used in most of these surveys were, in some instances, less sensitive for the detection of mycoplasma tissue-culture contaminants than seeding test material into a line of mouse lymphoma cells in vitro. This line showed cytopathic changes when infected with some mycoplasmas. The lymphoma cells also supported the growth of some non-cytopathogenic strains of mycoplasma from tissue cultures. A preliminary passage of the mycoplasmas in lymphoma cells produced sufficient enrichment to ensure their subsequent detection on solid media.

With the new awareness of mycoplasmas as potential contaminants many workers with tissue cultures tested them for the presence of these organisms. It soon became apparent that primary cultures prepared from a variety of human and animal tissues were rarely contaminated. Carski & Shepard (1961) found that each of two primary cultures from rabbit and hamster kidney were free of mycoplasmas. Barile, Malizia & Riggs (1962) found that only two out of 150 primary cultures of rhesus monkey kidney, and none of 60 rabbit kidney cultures, were contaminated. Rothblat (1960) noted that primary cultures from a variety of species were rarely contaminated and Herderschée, Ruys & van Rhijn (1963) found that 26 primary cell cultures were free from mycoplasmas. Others have made similar observations (Macpherson & Allner, 1960; Kraemer et al., 1963; Gori & Lee, 1964).

Although cell lines that are continuously propagated differ in a number of respects from primary and early passage cultures (Hayflick & Moorhead, 1961), the latter are just as capable as the former of supporting the growth of mycoplasmas, and the lower incidence of contamination in these cells is not due to some inherent resistance.
Mycoplasmas in tissue culture

Human diploid cells that have been in culture for several months may carry mycoplasmas (Herderschee et al., 1963). Primary cultures and cell cultures in early passage seem only to have had less opportunity of coming into contact with the sources of contamination.

MANIFESTATIONS OF MYCOPLASMA GROWTH IN CELLS

Inapparent infections

In many cultures found to be contaminated with mycoplasmas the changes in the cells have been minimal or inapparent (Rothblat, 1960; Carski & Shepard, 1961; Kraemer et al., 1963) and may be insufficient to cause the worker to seek their replacement or even to suspect there is anything amiss. Even heavy contamination may not be accompanied by damage to the cells or turbidity of the medium.

More commonly, some minor degree of cellular damage can be detected. Cells grow more slowly, are more granular, tend to come off the glass more readily, fail to form a continuous sheet, are more susceptible to trypsin, and produce more acid in the medium. Small necrotic foci may develop (Robinson et al., 1956). Some of these changes may pass unheeded if the worker receives the cells already contaminated and believes these manifestations to be characteristic of the cell line.

Cytopathic changes

In some cases a more striking cytopathic effect is caused by mycoplasma contaminants. Stim, Grace & Moore (1963) found a cytopathic agent with characteristics of a mycoplasma in a spontaneously degenerated culture of hamster tumour cells. It caused a cytopathic effect in primary bovine kidney cells but not in primary kidney cultures derived from some other species. As mentioned previously, Kraemer (1964) found that a cultured line of mouse lymphoma cells designated L5178Y responds to mycoplasmas isolated from a number of cell lines by undergoing lysis in a characteristic manner. At first some agglutination of the cells occurred, then cessation of metabolism was indicated by a rise in pH, followed finally by lysis and clearing of the cultures. The final pH of the infected cultures was about 7.5, whereas the controls, which had been incubated for the same time and were turbid with cells, had a final pH of about 6.9. Kraemer described instances in which attempted isolations of mycoplasmas from human diploid cell cultures produced no growth of colonies on agar medium but in which lytic infections were initiated when the same material was inoculated into L5178Y cells. Numerous colonies developed when medium from lysed cultures was plated on agar. He also observed that individual colonies picked from cultures prepared from contaminated strains of human diploid cells and inoculated into L5178Y cells could produce lytic infections, whereas other colonies, although establishing infections, were non-lytic. These two types could, on occasion, be isolated from the same cell culture and bred true when passaged. Non-lytic mycoplasmas caused no change in the growth rate of infected cells and could be recovered only sporadically from the cultures. In general it was found that contaminated cultures that gave rise to high yields of mycoplasmas on direct plating mostly contained non-lytic organisms.
that grew rapidly on agar. Cultures yielding few colonies on direct plating contained predominantly lytic mycoplasmas that grew slowly on agar.

When tested in the L5178 Y cells, the strains Campo, T-5, Eaton agent and seven unclassified human pharyngeal isolates were non-lytic. With lytic strains chronic infection could be established in HeLa, chick and mouse embryo and monkey kidney cultures, but no cytopathic effect was produced and the cultures grew as rapidly as the controls. On the other hand, the mouse ascites cell line P388D1 growing in vitro was rapidly lysed. It is of interest that both L5178 Y and P388D1 cells originated in DBA/2 mice. It is possible that cells of this mouse strain are unusually susceptible to mycoplasmas or may contain latent viruses that are 'ignited' by the mycoplasmas. As will be mentioned later, Kraemer suggests that their unusually high susceptibility may be related to their inability to substitute citrulline for the degraded arginine. In culture both these cell lines attach tenuously or not at all to the glass in stationary culture and thus may be vulnerable to changes in their cytoplasmic membranes mediated by mycoplasmas.

It has been shown that HeLa cells, known to be contaminated with mycoplasmas, grew satisfactorily when attached to glass but failed to grow in stirred suspension culture (Macpherson & Allner, 1960). If these cells were apparently cleared of mycoplasmas with neomycin they became capable of active division in suspension culture. Moore, Mount, Tara & Schwartz (1963) have also reported that several established lines of cells derived from human tumours failed to survive in suspension culture if they were contaminated with mycoplasmas. On the other hand, Brownstein & Graham (1961) were able to grow Earle's L strain of mouse fibroblasts in suspension culture although they were heavily contaminated with mycoplasmas. The colony-producing efficiency of the contaminated line on glass also remained unimpaired.

Experimental infections

A number of studies have been made on cultures deliberately infected with mycoplasmas (Hayflick & Stinebring, 1955; Wittler, Cary & Lindberg, 1956; Chanock et al., 1960; Fogh & Hacker, 1960; Castrejon-Diez, Fisher & Fisher, 1963; Carmichael, Fabricant & Squire, 1964). As in naturally occurring infections, there is a wide range of activity, from no detectable changes, through mild cytopathic effects, to destruction of the cells.

Microscopic appearance of infected cells

Microscopic examination of cells infected with mycoplasmas both by conventional histological methods (Hayflick & Stinebring, 1955; Shepard, 1958; Fogh & Fogh, 1964; Marmion & Goodburn, 1961) and by thin-section electron microscopy (Edwards & Fogh, 1960; Fogh & Hacker, 1960) reveals small masses of mycoplasmas within the cytoplasm and on the plasma membrane. Micro-colonies have been found occasionally in the nucleus (Carmichael et al., 1964). The majority of complete mycoplasma forms, both ovoid and filamentous, are predominantly found on the plasma membrane of the cells and, as will be discussed later, the high efficiency of specific anti-mycoplasma serum for decontaminating tissue cultures (Herderschee et al., 1963) suggests that the
main site for growth, or perhaps an essential phase of growth, is extracellular or on
the cell surface, since γ-globulins do not penetrate the cell membrane of viable cells.
Edwards & Fogh (1960) estimate the size of the ovoid forms found in thin sections to
be 540 by 300 mμ. Smaller viable forms undoubtedly exist in some strains of myco-
plasmas. Morowitz, Tourtellotte & Pollock (1963) found that 4 strains of mycoplasmas
could pass through a GS Millipore filter (0.22-μ pore size). Electron-microscope studies
of mycoplasmas grown on artificial medium indicate that some forms may be 125 mμ
in diameter (Morton, Lecce, Oskay & Coy, 1954) or even smaller (A. Howatson, personal
communication).

Biochemical basis of the cytopathic effect on cultured cells

The nutrition and metabolism of mycoplasmas in artificial medium has been
extensively reviewed (Smith, 1964). Studies of this kind are technically difficult since
no chemically defined medium has yet been devised even for the least exacting
members of this group. Present knowledge is based on the results obtained with
partially defined medium and with only a few strains of mycoplasma. Nevertheless,
it is clear that there is wide variation in the nutritional requirements and metabolic
activities of different strains. Studies on amino acid metabolism of mycoplasmas by
Smith (1955, 1957, 1960) have shown that certain strains metabolize glutamine,
glutamic acid and arginine. These are important metabolites for many strains of cells
in culture, and arginine is an essential amino acid for all strains so far studied.

The first indication that arginine depletion occurs in cultures infected with myco-
plasmas was provided by Powelson (1961), who used cell lines massively infected with
mycoplasmas isolated from bovine and avian sources. Kenny & Pollock (1963) showed
that the unusual appetite of the mycoplasmas for arginine was directly implicated in
their cytopathic effect on cells. Cultures of cell lines derived from human liver and
foetal intestine, when experimentally infected, grew poorly in medium supplemented
with 0.1 mM arginine. Infected cultures grew as well as mycoplasma-free cultures
when grown in 1 mM arginine. Mycoplasma populations in the latter two cases were
similar, indicating that the higher arginine level was not inhibitory to mycoplasmas.
Powelson (1961) has suggested that the alterations she demonstrated in the meta-
bolism of certain amino acids (including arginine) in infected cultures are dependent
on a mycoplasma/cell interaction. In the systems studied by Kenny & Pollock (1963)
this was not found to be so. Tissue-culture medium in which mycoplasmas had grown
and which was subsequently freed of mycoplasmas with kanamycin was found to be
deficient for cell growth. The addition of 0.1 mM arginine raised its growth-promoting
activity to that of the uninfected control medium.

Tissue-culture media are not always adequate for the growth of mycoplasmas.
Carski & Shepard (1961) found that solid media based on tissue-culture formulae did
not support the growth of mycoplasmas. Two media, (a) 40% human serum plus
60% Hanks’s balanced salt solution and (b) 20% human serum plus 10% bovine
foetal serum plus 70% balanced salt solution containing Eagle’s amino acids and
vitamins and 1 mM arginine, were both inadequate in this respect. The latter formula
as a liquid medium also failed to support the growth of mycoplasmas.
Medium that had been in contact with HeLa cells for 3 days was also inadequate. In the presence of HeLa cells growing in this medium mycoplasmas grew readily to high titre. Powelson (1961) also found that medium 199 plus 2% horse serum did not support mycoplasma growth. However, Fogh, Hahn & Fogh (1965) found that mycoplasmas isolated from several tissue cultures were capable of growing in cell-free tissue-culture medium. Growth of the mycoplasmas was enhanced by the addition of yeast extract.

In a carefully controlled series of experiments Kraemer (1964) has shown that the lysis of the mouse lymphoma cell line _L5178Y_ induced by certain strains of mycoplasmas is due to depletion of arginine in the medium, and these cells may be protected from the lytic action of the mycoplasmas by maintaining a high level of arginine in the medium. Non-lytic strains of mycoplasmas that multiply to high titre and establish chronic infections do not deplete the arginine in the medium to a significant extent. Unlike the system described by Kenny & Pollock (1963), Kraemer found residual arginine-depleting activity in mycoplasma-freed medium. Kraemer suggests that the marked susceptibility of the _L5178Y_ mouse lymphoma cells to the lytic agents may be due to their inability to substitute citrulline for the degraded arginine. The scheme suggested by Schimke & Barile (1963) would not support this view. They studied the stoichiometry of arginine breakdown by mycoplasma-infected HeLa cultures, and proposed the following pathway (which is the arginine dihydrolase system first described by Hills (1940) in _Streptococcus_) to explain their findings:

\[
\text{Arginine deiminase} \rightarrow \text{Citrulline} + \text{NH}_3 \quad \text{Ornithine} \quad \text{Pi transcarbamylase} \\
\text{CO}_2 + \text{NH}_3 = \text{ATP} \quad \text{Carbamyl phosphate} + \text{ornithine}
\]

The breakdown of arginine is not due to arginase activity since neither mycoplasma-contaminated nor mycoplasma-free HeLa cells have measurable urease activity, and this enzyme would be necessary to account for the absence of urea formation during arginine degradation. Extracts of infected HeLa cells, as well as extracts of mycoplasmas in cell-free broth, rapidly converted arginine to ornithine. Since arginine deiminase activity is not present in animal tissues, Barile & Schimke (1963) have suggested that the demonstration of this activity in tissue cultures may be used as a rapid method for the detection of mycoplasma contamination. This will be discussed later.

Depletion of other metabolites in tissue-culture media by contaminating mycoplasmas undoubtedly occurs and may result in cell destruction (Fogh _et al._, 1965), but as yet there has been no report of specific effects (other than that concerning arginine) which may be implicated in causing cell degeneration. Powelson (1961) noted that the glutamine concentration in mycoplasma-infected chick heart fibroblasts was reduced in comparison with the control. However, Kenny & Pollock (1963) found that reduction of the glutamine level in infected cultures from 2 to 0.2 mM did not cause detectable alterations in cell growth.
Mycoplasma in tissue culture

One of the most important activities attributed to mycoplasma contaminants of cultured cells was recognized by Hakala, Holland & Horoszewicz (1963). They had previously found that a number of cell lines, including HeLa, when grown in the presence of amethopterin, can utilize thymidine, thymidylic acid and 5-methylcytosine deoxyribonucleoside as sources of DNA thymidine (Holland et al., 1963). Growth also occurred when this medium contained 5-bromodeoxyuridine in place of thymidine. However, they found that growth of one strain of HeLa cell (subsequently found to be contaminated with mycoplasmas and designated HeLa/PPLO) was not supported by these deoxyribonucleosides in amethopterin-containing medium. The cells had also become much more resistant to inhibition by 5-fluorodeoxyuridine. Growth of HeLa/PPLO in amethopterin medium was not supported by the addition of 5-bromodeoxyuridine or 5-iododeoxyuridine.

When HeLa/PPLO cells were incubated with thymidine this was rapidly broken down. The observation that 5-fluorouracil and 5-fluorodeoxyuridine were equally powerful inhibitors of HeLa/PPLO, while for another cell line (S-180) the nucleoside was 43 times more inhibitory than the free base, suggested that 5-fluorodeoxyuridine was also being cleared by HeLa/PPLO. This activity was indeed demonstrated. Since HeLa/PPLO was found to be more sensitive to 6-mercaptopurine medium supplemented with folic acid than the HeLa cell line studied previously, they concluded that the enzyme system for metabolizing hypoxanthine was not impaired. Studies with mycoplasmas isolated from the HeLa/PPLO cells and also with four other strains of mycoplasmas from various sources showed that they all were able to cleave thymidine to thymine. The substrate specificity of the mycoplasma enzyme indicated that it is similar to the pyrimidine nucleoside phosphorylase isolated from horse liver (Friedkin & Roberts, 1954). From these results it is clear that any studies involving the metabolism of deoxyribonucleosides in mammalian cells must take into account the possible contribution of mycoplasma contaminants. Two recent papers emphasize this point. Randall, Gafford, Gentry & Lawson (1965) found that in HeLa cell cultures infected with mycoplasmas the host-cell DNA is unstable, since acid-soluble radioactive label, previously incorporated in the cell DNA, may be detected in the medium. This characteristic can be transmitted to mycoplasma-free cultures of L cells by infecting them with mycoplasmas from the HeLa cells. Nardone, Todd, Gonzalez & Gaffney (1965) found that contamination of L-cell cultures by mycoplasmas inhibited incorporation of tritiated thymidine and uridine. Autoradiographs of contaminated cultures were characterized by exposed silver grains at the cell margins. Treatment of the cells with kanamycin restored normal nucleoside incorporation.

Other effects on the nutrition of tissue cultures

The addition of yeast extract and also Staphylococcus culture-medium filtrates increases the cytopathic effect of mycoplasmas in tissue culture, probably by stimulating the growth of the organisms (Wittler et al., 1956; Kenny & Pollock, 1963; W. House (personal communication)). Edward (1947) found that yeast extract improved the growth of mycoplasma in artificial media. The factor or factors in yeast extract responsible for this enhancement have not been characterized.
The reducing conditions in culture may also affect the growth of some mycoplasmas. Herderschee et al. (1963) found that a mycoplasma-free line of HeLa cells failed to support the growth of *Mycoplasma salivarium*, a strain that requires anaerobic conditions for optimal growth. If, however, 5% Filde's extract (a peptic digest of sheep's blood) was added *M. salivarium* grew well and gave a cytopathic effect. Filde's extract by itself did not affect the growth of the cells. The authors suggest that the effect may be due to catalase in the extract. The addition of yeast extract and/or Filde's extract to tissue cultures may be a useful preliminary to testing these cultures for mycoplasmas, since a low-grade infection may be stimulated.

**MYCOPLASMAS IN RELATION TO VIROLOGY AND ONCOLOGY**

**Similarities between mycoplasmas and viruses**

Since some mycoplasma strains produce cytopathic changes in cultured cells it is important to recognize this fact when attempting to isolate infectious agents in tissue culture. A 'filter-passing', cytopathogenic, transmissible agent may be either a virus or a mycoplasma. As was mentioned previously, some mycoplasma forms pass through 0.22-μ Millipore filters (Morowitz et al., 1963) and the smallest particle capable of independent existence may be about 125 μ (Morton et al., 1954). The similarity of mycoplasma to some viruses, especially the myxoviruses, extends to: their appearance in the electron microscope; sensitivity to ether and chloroform; inhibition of growth by specific antiserum; interference with virus replication *in vitro* (Rouse, Bonifas & Schlesinger 1963; Somerson & Cook, 1965; Pontén, to be published); ability of some strains to haemagglutinate (Adler, 1954) and also to give rise to haemadsorption on infected cells (Berg & Frothingham, 1961); their resistance to some antibiotics; and the induction of chromosome aberrations (Paton, Jacobs & Perkins, 1965; Fogh & Fogh, 1965).

Also, like the oncogenic viruses of polyoma and Rous sarcoma, some mycoplasmas of human origin have been shown to mediate changes in the *BHK 21* line of hamster fibroblasts that enable the cells to form colonies in agar suspension culture and in some cases also to undergo morphological transformation (Macpherson & Russell, to be published).

**Association of mycoplasmas with neoplastic tissue**

In diseases in which the aetiological agent may be suspected of being a virus, on the grounds that a similar syndrome has been shown to be due to a virus in other species, the temptation to claim virus-like effects with isolated agents should be particularly avoided. Agents isolated from the bone marrow of leukemic patients were originally thought to be viruses, mainly because of the cytopathic effect they had for human embryonic kidney cells *in vitro* (Negroni, 1964), but these agents were subsequently found to be mycoplasmas by Grist & Fallon (1964). Their suspicions were aroused when the agents were found to be cytopathogenic for cell cultures derived from a wide range of animal species. They were also able to culture typical mycoplasmas from the degenerating cultures and were able to show that the cytopathic
Mycoplasmas in tissue culture

Effect of mycoplasma contamination on virus growth in tissue culture

In a number of instances the growth of viruses in tissue culture has been found to be unaffected by the presence of large numbers of contaminating mycoplasmas. Herderschë et al. (1963) found that seven recently isolated polio viruses of type 1 and the Sabin strain B1-3-F1 grew to approximately the same titre in a line of human embryonic lung fibroblasts both in the presence and absence of contaminating mycoplasma (M. hominis type 1). Gori & Lee (1964) examined the sensitivity of a number of mycoplasma-contaminated cell lines to vaccinia, poliovirus type 1 and measles virus, both before and after the contaminating organisms had been eradicated. Those with low levels of contamination (i.e. less than 100 colony-producing organisms per 10^6 cells) showed no difference in maximum yields of virus obtained. In heavily contaminated cell lines the yield of virus was slightly improved after treatment. Similar results were obtained by Sever (see Gori & Lee, 1964) with rubella virus. Brownstein...
Macpherson & Graham (1961) found that the single-burst yields of EMC virus from mycoplasma-contaminated L cells was lower than that obtained with this virus in uncontaminated cells. They suggest the difference may have been due to the presence of the contaminating organisms. O’Connell, Wittler & Faber (1964) found that an unidentified mycoplasma enhanced the cytopathogenic effect of a latent simian virus when it was added to green monkey kidney cultures.

Pollock, Treadwell & Kenny (1963) have suggested that since inhibition by ammonium ions of influenza virus formation has been demonstrated (Eaton & Scala, 1961; Jensen, Force & Unger, 1961) the presence of mycoplasmas may have an effect on virus multiplication, since some strains produce ammonia. A striking example of mycoplasma interference of virus growth has been provided by Somerson & Cook (1965). They found that the growth of Rous sarcoma virus (RSV) was inhibited in cultures of chick embryo cells infected by a strain of *M. orale*, a commensal organism of the upper respiratory tract of man. This mycoplasma strain, originally isolated from human diploid fibroblast cultures, was cytopathogenic but lost this property when passaged on solid medium. Cytopathogenicity could be regained by passage in chick embryo fibroblasts. Both cytopathogenic and non-cytopathogenic substrains caused inhibition of RSV focus formation. Suppression of Rous-associated virus (RAV) was also demonstrated by failure to detect avian leukemia complement-fixing antigen in mycoplasma-infected chick-embryo fibroblasts inoculated with RSV. The inhibitory effect was not extended to a strain of influenza B virus.

Similar results have been obtained by Pontén (1965) with a transmissible non-cytopathic organism derived from a culture of human tumour cells. Although he was unable to culture mycoplasmas from the culture fluids that initiated resistance to RSV, the sensitivity of the agent to antibiotics indicated that it was probably a mycoplasma. Macpherson & Pontén (unpublished data) found that chicken fibroblasts exposed to *M. hominis* type 1 mycoplasmas at a multiplicity of 1 colony-forming unit per cell subsequently failed to develop any foci when challenged with RSV. In this case the mycoplasma infection was quite inapparent. Inhibition of RSV and RAV by mycoplasmas could clearly cause considerable confusion in the interpretation of the interplay of these viruses.

Schlesinger (1961) found that adenovirus plaque-forming efficiency was enhanced when the arginine concentration in the medium was increased. This effect was subsequently correlated with the presence of mycoplasmas in the cultures (Rouse et al., 1963). Type 2 adenovirus growing in KB or other cells was found to have an absolute requirement for arginine in the medium. As stated above, some mycoplasmas rapidly deplete arginine in the medium of the cultures they contaminate. It is likely that other viruses, such as herpes simplex, that are dependent on an adequate supply of arginine for maturation, may be similarly affected by mycoplasma contamination of the cells in which they are grown.
DETECTION OF MYCOPLASMA CONTAMINATION

Changes in the gross appearance of cultures

The spectrum of morphological alterations that may occur in mycoplasma-contaminated cultures has already been described. It is important to note that in some cultures these changes may be so small as to pass unnoticed. However, in many other instances there are fairly obvious indications that the cells are growing suboptimally.

Microscopic appearance of infected cells

By fixing and staining cells grown on cover glasses it is often possible to detect small round ovoid or filamentous structures stained with the specificity of DNA on the cells. Intensified Giemsa-staining described by Marmion & Goodburn (1961) has been used to reveal mycoplasma contamination of cells in culture (Butler & Leach, 1964; see Fig. 1). Eaton, Farnham, Levinthal & Scala (1962) found that May–Grünwald–Giemsa stain demonstrated M. pneumoniae in cultures of human amnion and human embryonic lung. Fogh & Fogh (1964) recommend a hypotonic treatment, air-drying and orcein-staining procedure based on the methods commonly employed in karyological analysis. Mycoplasmas are easily seen in the extended cytoplasm of these cells. In Fig. 2 the individual mycoplasmas are shown stained by Giemsa in a chromosome preparation of BHK21 hamster cells. It is doubtful if any staining method would be adequate to detect low-grade contamination of cells and it would certainly be unwise to rely solely on staining methods for the routine examination of cultures for contamination. Nevertheless, it is a worthwhile preliminary and should reveal gross contamination. The use of direct fluorescent antibody methods on infected cells suffers from the same defect and also from the fact that, although many current contaminants are of the same serological type (see below in section on typing), there are exceptions which would be missed if a mono-specific antiserum were used. The use of multivalent labelled antisera would not be practical. It may also be noted here that cells heavily contaminated with mycoplasmas adsorb fluorescent globulin non-specifically on their surface (Macpherson, unpublished observation).

Methods based on biochemical reactions

As previously stated, many strains of mycoplasmas possess arginine deiminase and thymidine-cleaving activity. The enzymes catalysing these reactions have not been found in animal cells. Colorimetric methods for their detection have been devised and suggested as suitable for the detection of mycoplasma contamination of tissue-cultured cells.

Barile & Schimke (1963) have described a method for the assay of arginine deiminase activity in cell extracts. The enzyme is detected by formation of citrulline from arginine at pH 6.5, the citrulline being determined colorimetrically. Barile & Schimke estimated the total number of organisms required for a positive reaction to occur in their test at between $10^5$ and $10^6$. This estimate was made both on broth cultures and on contaminated cell-culture lysates. Contaminated cell cultures examined generally contained at least 10 times more mycoplasmas than specified for the test.
They believe the sensitivity of the test could be improved by extending the incubation period for the enzyme reaction. The results obtained were in agreement with cultural procedures in 73 cases examined. Since some strains of bacteria also possess arginine deiminase activity, potential false results with the arginine deiminase method for mycoplasma contamination could be obtained in the presence of these bacteria. In practice activity was found only in association with cells contaminated with mycoplasmas. However, as bacterial infection is equally undesirable, its detection by this method is no disadvantage.

Horoszewicz & Grace (1964) make use for detection of the mycoplasmas’ ability to cleave thymidine. The amount of free deoxyribose resulting from the degradation of thymidine is measured. Of 42 strains grown in broth culture, 38 gave positive reactions. The demonstration of activity requires disruption of the organisms. In the cell culture test from $10^9$ to $10^7$ disrupted cells are necessary. Uninfected L-029 cells produced only $0.117 \mu$moles of deoxyribose per min per mg of protein under test conditions, whereas the same cells infected with a mycoplasma (strain 880) produced 5-50 times more. When 73 cell cultures of 20 different cell lines were examined for thymidine-cleaving activity, 46 cultures were positive and 27 were negative. The results agreed with isolations made on artificial media. However, House (personal communication) has found that thymidine-cleavage activity may be low in some cultures heavily contaminated with mycoplasmas.

**Isolation of mycoplasmas in artificial media**

A variety of artificial media have been successfully used for cultivation of mycoplasmas. Usually they are meat-infusion or digest broths to which have been added 10-20% serum and 5-10% yeast extract. The yeast extract described by Hers (see Lemcke, 1965) has been found to be of particular value in the stimulation of mycoplasma growth (Lemcke, 1965; House, personal communication). For solid media a soft gel is produced by the addition of agar or agarose to the enriched broth. The grade of agar is important since some types of agar contain inhibitors (Lynn & Morton, 1956). ‘Bacto’ PPLO agar (Difco) is usually suitable, although some organisms fail to grow on this medium (Randall et al., 1965).

Some difficulty may be experienced in identifying mycoplasma colonies by those unfamiliar with their morphology. Typically they are like fried eggs in that they have an inner dense region and an outer less-dense area when examined by low-power microscopy. A microscope with a total magnification of 100 diameters is suitable. A number of artifacts may simulate mycoplasma colonies (Hayflick, 1965). Of these, disrupted tissue-cultured cells and accretions of calcium and magnesium soaps from the medium (Brown, Swift & Watson, 1940) cause most difficulty. A preliminary culture in broth inoculated with test cells and medium followed by plating on agar eliminates the former difficulty and will increase the mycoplasma population. Since mycoplasmas usually grow into the agar a useful test is to attempt to scrape a suspected colony from the surface of the plate. The central core of a colony of mycoplasmas will remain behind but artifacts or pseudoccolonies will be dislodged. However, on first isolation from tissue culture some mycoplasmas form atypical colonies (Herderschee
Mycoplasmas in tissue culture

et al., 1963) without the typical fried-egg appearance of strains well adapted to growth on solid medium. The concentration of the agar will also influence the appearance of the colony. If the agar is too hard the colonies will be entirely on the surface and may be reduced in size and number (Macpherson, unpublished observation).

It is therefore important to ensure that desiccation of the medium does not occur during incubation. Incubation under anaerobic conditions (95 % nitrogen with 5 % carbon dioxide) may be superior to aerobic conditions for the isolation and growth of some mycoplasmas from tissue culture (Barile, Yaguchi & Eveland, 1958; Butler & Leach, 1964; Herderschee et al., 1963). Cultures should be made both aerobically and anaerobically for initial isolation. Incubating cultures in closed jars that have been flushed out with 5 % carbon dioxide in nitrogen is adequate for decreasing the oxygen content of the atmosphere. A closed container prevents the agar medium from drying and stiffening.

When there is doubt that a structure is a mycoplasma colony, a further check may be made by attempting to stain it with Dienes's stain (Dienes & Weinberger, 1951). This stain (a mixture of maltose, methylene blue and azure II) is taken up by mycoplasma and bacterial colonies. The latter decolorize the stain after about 30 min but the colonies of mycoplasmas retain the stain. However, colonies of dead bacteria may retain the stain. Impressions of colonies may also be fixed on to a slide by cutting out a block of agar medium bearing the colonies and inverting it on a microscope slide. The slide is then immersed in Bouin's fixative for several hours and, after the removal of the agar block, washed and then stained with Giemsa. The stained impression of the colony has a characteristic vacuolated appearance within which the granular large 'bodies' of the mycoplasma may be distinguished using high-power microscopy. These vary in staining intensity and range from 0.3 to 1.0 μ in diameter.

The use of the enriched culture medium employed for the isolation of mycoplasmas has the added advantage that it is also capable of supporting the growth of bacteria which would normally be missed because of their inability to grow on standard bacteriological media. In the course of screening cultures for mycoplasma contamination, Coriell (1960) detected persistent infection of a culture with diphtheroid bacteria. The bacteria produced no gross microscopic change in the tissue cultures nor did they grow in thioglycollate broth or standard blood agar plates. If antibiotic-free tissue-culture medium was used the organisms grew out and destroyed the cells. Barile & Schimke (1963) also found low-grade bacterial contamination (100–1,000 bacteria per ml) in 20 out of 73 cell-culture media tested.

SOURCES OF MYCOPLASMA CONTAMINANTS

There has been much speculation about the source of mycoplasma contaminants in tissue culture. No single explanation satisfactorily accounts for all contaminations. However, it is important to detect the main sources of contamination to enable prophylactic measures to be taken. As will be discussed later, there is good evidence that the majority of contaminations result from droplet spray infections from cultures already contaminated, but the source of primary contaminations is perhaps less
certain. The following possibilities suggest themselves: the tissue used to initiate the culture was infected with mycoplasmas; a constituent of the medium contained mycoplasmas; the mycoplasmas are in fact L-forms of contaminating bacteria, converted in vitro; and mycoplasmas from man have been introduced during processing of the cultures.

Serotypes of mycoplasma contaminants in cultures

The most useful information for the tracing of micro-organisms is provided by phage or serological typing. This latter technique may be used for mycoplasmas, and determination of serotypes is an obvious first step in recognizing their source. The evidence obtained by such surveys strongly suggests the last possibility offered above as the most important mode of primary contamination, since mycoplasmas in tissue culture belong almost exclusively to human serotypes.

Several methods of serological typing of mycoplasmas may be employed and good qualitative correspondence occurs in results obtained by different methods. In Table 1 the results of several typing surveys are listed along with references. Clearly *M. hominis* type 1 is the predominant organism. Organisms with this serotype have been isolated from the genitalia of man and less frequently from the upper respiratory tract. In the latter situation *M. hominis* type 1 is not the commonest commensal mycoplasma as judged by isolations made on artificial medium (D. Taylor-Robinson, personal communication). If the upper respiratory tract is the primary source of most contaminations then one might expect the common types of commensals (e.g. *M. salivarium*) to

Table 1. Serological typing of mycoplasmas isolated from tissue cultures

<table>
<thead>
<tr>
<th>No. of isolates examined</th>
<th>Method of typing</th>
<th>Distribution of serotypes (number)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>32</td>
<td>Growth inhibition</td>
<td><em>M. hominis</em> type 1 (31)</td>
<td>Herderscheé <em>et al.</em>, 1963</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>M. orale</em> (1)</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>Complement fixation</td>
<td><em>M. hominis</em> type 1 (11)</td>
<td>Lemcke, 1964; a, b</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>M. orale</em> (5)</td>
<td></td>
</tr>
<tr>
<td>49</td>
<td>Fluorescent antibody</td>
<td>Not typed but all interacted with</td>
<td>Barile <em>et al.</em>, 1962</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>M. hominis</em> type 1 (49)</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Agglutination and growth inhibition</td>
<td><em>M. hominis</em> type 1 (4)</td>
<td>Bailey <em>et al.</em>, 1961</td>
</tr>
<tr>
<td>5</td>
<td>Growth inhibition</td>
<td><em>M. hominis</em> type 1 (4)</td>
<td>Clyde, 1964</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Patt strain (1)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Growth inhibition</td>
<td><em>M. hominis</em> type 1 (1)</td>
<td>Edward, 1960</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>M. gallisepticum</em> (1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Unidentified (2)</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>Complement fixation</td>
<td>All same but not <em>M. hominis</em></td>
<td>O'Connell <em>et al.</em>, 1964</td>
</tr>
<tr>
<td></td>
<td></td>
<td>type 1 or 2 or <em>M. salivarium</em></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Complement fixation</td>
<td>'Similar to human genital PPLO'</td>
<td>Collier, 1957</td>
</tr>
<tr>
<td>1</td>
<td>Complement fixation and growth inhibition</td>
<td>Not <em>M. hominis</em> type 1 or 2, salivarium or Innes</td>
<td>Butler &amp; Leach, 1964</td>
</tr>
</tbody>
</table>
be the commonest tissue-culture contaminants. One possible explanation why this is not so is that these strains do not adapt to tissue-culture conditions as readily as *M. hominis* type 1. Herderschee et al. (1963) provide evidence to support this belief. They found that under normal culture conditions *M. salivarium* failed to initiate an infection in HeLa cells and disappeared after 10 days. If 5% Fildes' extract (which contains catalase) was added to the medium *M. salivarium* grew well and was cytopathic. Good evidence that culture-to-culture spread occurs has been provided by O'Connell et al. (1964), Herderschee et al. (1963), Hakala et al. (1963), and Randall et al. (1965). O'Connell et al. (1964) found that aerosols generated during trypsinization of infected cultures contained mycoplasmas. They presented evidence that a single mycoplasma cell is capable of initiating infection in a tissue culture and showed that mycoplasmas could be transferred from one culture to another through the use of a common burette for dispensing medium. The organisms they isolated from 15 antibiotic-free tissue cultures were closely related serologically, but antisera prepared against them failed to produce significant reactions with a number of stock culture strains including *M. hominis* types 1 and 2 and *M. salivarium*. One isolate was found to cross-react with an unclassified strain of mycoplasma isolated from the throat of a child with severe pharyngitis, again suggesting that the upper respiratory tract may have been the origin in this case.

Another strain of mycoplasma isolated from tissue cultures (Herderschee et al., 1963; Lemcke, 1964a) has been shown to have serological identity with *M. orale*, another commensal of the upper respiratory tract of man (Lemcke, 1964b). Thus with a few exceptions the organisms found in tissue cultures can be identified as having serological cross-reactivity with strains from the upper respiratory tract of man. There now seems little doubt that this is the primary source of contaminants.

However, other possible sources of contamination deserve consideration. We have seen that primary cultures of normal tissue are rarely contaminated but cultures of malignant tissue frequently contain mycoplasmas (Grace et al., 1963). Many attempts to demonstrate mycoplasmas in the serum employed for tissue culture have failed, but the fact that small forms of some mycoplasmas may pass through 0.22-μm Millipore filters makes it possible for them to survive serum-processing if they are present on occasion. If the serum retains its growth-promoting activity after heating at 56 °C for the particular cells under study then it would seem to be a worthwhile additional safeguard against contamination, although Collier (1957) found that some mycoplasmas survived in human serum deliberately seeded and heated at 55 °C for 1 h.

Further evidence that medium is not an important source of contamination is provided by the evidence of several workers who found that contaminated cells, once cured by treatment with antibiotics, may remain free of mycoplasmas for many months after the antibiotics have been removed (Pollock et al., 1960). Chick-embryo extract is a potential source of contamination, although it is not often used now. Mycoplasmas have been isolated from chick embryos (van Herick & Eaton, 1945) and they would certainly survive the processing procedures usually adopted for the preparation of extracts. Edward (1960) has isolated an avian mycoplasma (*M. gallisepticum*) from a tissue culture and suggested it may have been derived from chick-embryo extract.
The notion that most mycoplasmas in tissue are in fact L-forms, derived from bacteria that had contaminated the cultures and then undergone conversion due to contact with penicillin or some other constituent of the medium, is no longer acceptable. It is very unlikely that the contaminating bacteria would convert so regularly into organisms with the serological specificity of *M. hominis* type 1. However, it has been shown that 'mycoplasmas' in some cultures are capable of giving rise to colonies of corynebacteria (Macpherson & Allner, 1960; Carter & Greig, 1963) and Gram-negative rods (Holmgren & Campbell, 1960) under appropriate cultural conditions. It is uncertain whether these instances represent cases of L-form conversion in tissue-culture or whether the contamination was with 'mycoplasma' of human origin and these reverted to their bacterial form when cultured on artificial medium after their sojourn in tissue culture. There is of course much support for the idea that some, if not all, mycoplasma are of bacterial origin (Dienes & Weinberger, 1951; Pease, 1965) but a discussion of this possibility is beyond the scope of this review.

**CONTROL OF MYCOPLASMA CONTAMINATION**

Although the adage 'prevention is better than cure' certainly applies to mycoplasma contamination of tissue cultures, situations arise in which it is worth while attempting to decontaminate an infected culture. Most reported methods involve the use of antibiotics, but other means have been suggested.

Hayflick (1960) has shown that maintenance of contaminated HeLa and L cells at 41 °C for 18 h kills some strains of mycoplasma differentially, without damaging the cells beyond recovery. Other workers have found this method to be unsatisfactory because either the cells failed to recover or the mycoplasmas were not inactivated (Herderschee et al., 1963; Balduzzi & Charbonneau, 1964).

Another approach is that described by Kenny & Pollock (1963) and Herderschee et al. (1963) who made use of the inhibitory effect of specific antiserum on homologous mycoplasmas (Edward & Fitzgerald, 1954). They found that passage of the cells in medium containing the appropriate antiserum results in the disappearance of the mycoplasmas. The method has some serious disadvantages. Antiserum must be specific for the mycoplasma strain and, although *M. hominis* type 1 is most frequently isolated from tissue cultures, other strains of human mycoplasmas and some of unknown origin (Butler & Leach, 1964) are being isolated as tissue-culture contaminants with increasing frequency (Taylor-Robinson, personal communication). It is doubtful if a sufficient range and volume of potent specific antisera could be obtained for this purpose. The method may have special applications, since it is the least cytotoxic and therefore the least selective to the cell population, in cases where damage to a valuable cell line must be avoided. The contaminating organisms could be isolated and the cells stored whilst antiserum was being prepared.

Antibiotic therapy offers the best chance of successful decontamination. A number of antibiotics are known to be effective against mycoplasmas in tissue culture at levels which are non-toxic or relatively non-toxic to the cells harbouring them. Successful decontamination of cultures has been reported using neomycin (Macpherson &
Mycoplasmas in tissue culture

Allner, 1960), tetracycline (Carski & Shepard, 1961), kanamycin (Pollock et al., 1960; Fogh & Hacker, 1960; Herderscheé et al., 1963), 7-chlorotetracycline (Gori & Lee, 1964), and a mixture of chloramphenicol and novobiocin (Balduzzi & Charbonneau, 1964). The recommended concentration of antibiotic and the duration of the treatment vary considerably in different reports. Factors which will influence the result include the strain of contaminating mycoplasmas, the strain of cells, the composition of the medium, the frequency of medium changes, the pH at which the culture is normally maintained, and the method of subculturing. The most useful guide to the selection of suitable antibiotics is provided by the work of Perlman & Brindle (1965) who studied the toxicity of antibiotics for cells in culture and the sensitivity of 8 strains of mycoplasmas against 40 antibiotics. Their work is summarized in Tables 2 and 3.

Unfortunately, treatment of contaminated cultures is not a straightforward procedure of selecting an antibiotic effective at a non-cytotoxic level. Antibiotics have variable stabilities in tissue-culture media and may be rapidly inactivated at 37 °C (Table 3). Also, antibiotics are less effective against intracellular than against free bacteria (Murat, Stinebring, Schaffer & Lechevalier, 1959) and presumably are less effective against intracellular mycoplasmas. Although mycoplasmas may grow only on the surfaces of cells and their appearance in the cytoplasm may be the temporary result of pinocytosis, their sojourn there may render them resistant to antibiotics that fail to penetrate the plasma membrane or do so only poorly. Finally, and most importantly, mycoplasma strains may become resistant to certain antibiotics. This has been found with some tissue-culture contaminants for kanamycin (Macpherson, unpublished data) and tetracycline and chloramphenicol (Balduzzi & Charbonneau, 1964). It is possible that resistance to most antibiotics would eventually be gained by mycoplasmas grown in sub-inhibitory levels of them, and thus care must be exercised in using suitable antibiotics in the most effective way. The development of antibiotic-resistant variants is to be avoided at all costs, since culture-to-culture spread is apparently common.

A preliminary to work with any cell line or cell strain should be the preparation of a master seed, stored with appropriate additives (Wallace, 1964) in liquid nitrogen or

Table 2. The effect of antibiotics on mycoplasma and on tissue-cultured cells
(From Perlman & Brindle, 1965.)

| Antibiotics essentially inactive against mycoplasma in vitro | Amphotericin, Bacitracin, Benzylenicillin, Candidin A, Cycloheximide, Cycloserine, Etruscomycin, Filipin, Griseofulvin, Nystatin, Oleandomycin, Patulin, Polyphycin, Ristocetin, Streptomycin, Trichomycin, Vancomycin, Vernamycin A and B, Viomycin |
| Antibiotics active against mycoplasma but too cytotoxic for use in tissue culture | Carbomycin, Dactinomycin, Streptothricin, Stendomycin, Thiostrepton |
| Antibiotics useful in controlling mycoplasma contamination in tissue culture | See Table 3 |
Table 3. Antibiotics useful in controlling mycoplasma contamination in tissue cultures
(From Perlman & Brindle, 1965.)

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Stability in tissue culture media*</th>
<th>Concentration showing marked cytotoxicity (mcg/ml)</th>
<th>Minimum concentration inhibiting mycoplasma in artificial medium (mcg/ml)</th>
<th>Concentration recommended for controlling mycoplasma in tissue cultures† (mcg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloramphenicol</td>
<td>High</td>
<td>30</td>
<td>15</td>
<td>30</td>
</tr>
<tr>
<td>7-Chlortetracycline</td>
<td>Very low</td>
<td>80</td>
<td>40</td>
<td>100</td>
</tr>
<tr>
<td>6-Demethyl-7-chlortetacycline</td>
<td>High</td>
<td>15</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>Moderate</td>
<td>300</td>
<td>15</td>
<td>50</td>
</tr>
<tr>
<td>Fusidic acid</td>
<td>High</td>
<td>40</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>High</td>
<td>3000</td>
<td>1</td>
<td>200</td>
</tr>
<tr>
<td>5-Hydroxytetracycline</td>
<td>Moderate</td>
<td>35</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>Hygromycin B</td>
<td>Moderate</td>
<td>300</td>
<td>15</td>
<td>50</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>Very high</td>
<td>1000</td>
<td>25</td>
<td>200</td>
</tr>
<tr>
<td>Neomycin B</td>
<td>Very high</td>
<td>3000</td>
<td>15</td>
<td>50</td>
</tr>
<tr>
<td>Novobiocin</td>
<td>Low</td>
<td>200</td>
<td>10</td>
<td>50</td>
</tr>
<tr>
<td>Paromomycin</td>
<td>High</td>
<td>5000</td>
<td>20</td>
<td>50</td>
</tr>
<tr>
<td>Spiramycin</td>
<td>Moderate</td>
<td>1000</td>
<td>1</td>
<td>50</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>Moderate</td>
<td>35</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>Tylosin</td>
<td>Moderate</td>
<td>300</td>
<td>1</td>
<td>10</td>
</tr>
</tbody>
</table>

* Stability scale: half-life of 2 days, very low; 4 days, low to moderate; 8 days, very high.
† Recommended on basis of 3-day incubation period between changes of medium.

It is advisable to determine the maximum tolerated doses for the recovered cells of a number of antibiotics effective against mycoplasmas. Since the likelihood of a mycoplasma strain gaining resistance simultaneously to structurally and biochemically different antibiotics is low, combinations of such antibiotics should also be tested for their toxic level. Great care should be taken to ensure that the master seed is free of mycoplasmas by testing and by treatment with combinations of antibiotics. The levels used for the hamster line BHK21 (Macpherson & Stoker, 1962) in the author's laboratory are: kanamycin, 1 mg/ml; novobiocin, 50 μg/ml; and chlortetracycline, 5 μg/ml. These antibiotics are incorporated into medium which is changed every 2 days for 6 days on an initially sparse culture. Treatment is followed by repeated testing for mycoplasmas by as many means as possible but especially by culturing both directly on to solid medium and also following an intermediate passage in broth.

The observations of Gori & Lee (1964) suggest that conventional antibiotic treatment may at best be capable only of depressing a mycoplasma infection and may fail to eliminate the organisms completely even after prolonged treatment. This was so when a near cytotoxic level of tetracycline was used in tissue-culture medium for up to 5 months. To eradicate mycoplasmas from cell lines they subjected the cells to
treatment for a few minutes with very high concentrations of aureomycin, kanamycin, and chloramphenicol in water. This was followed by cultivation of the cells in medium with a lower concentration of antibiotics before final omission of the antibiotics for 2 medium changes. Cell disintegrates were then tested for mycoplasma on solid medium. If a positive isolation of mycoplasmas was obtained, the cell culture was subjected to another cycle of hypotonic antibiotic treatment. Only aneuploid cell lines like HeLa survived this treatment and it was quite unsuitable for diploid cell lines.

If the observations of Gori and Lee are confirmed in other systems and complete eradication of mycoplasmas requires the drastic treatment they describe, then the use of short-term cultures derived from replicates of a frozen master seed will become desirable in order to reduce the possibility of contamination. This will be especially important for cell strains that do not survive the treatment they prescribe. It is also clear that a general improvement of aseptic techniques for tissue-culturing will result in a lower incidence of contamination.

CONCLUSIONS

Although it is apparently widely appreciated that cultures of mammalian cell lines may become contaminated with mycoplasmas, it is also clear that few workers know if their cultures are contaminated or indeed ever take steps to find out. Studies with infected cells in the fields of virology, biochemistry, immunology and oncology are subject to profound misinterpretations, and mycoplasma infections may remain undetected for many months if surveillance is not carried out routinely.

In experiments in which mycoplasma contaminations could conceivably produce a spurious result, a study of the consequences of deliberately infecting the cell system with mycoplasma (e.g. a common tissue-culture contaminant like M. hominis type 1) could be an informative control.

Of particular interest are the recent observations that cell-associated mycoplasmas may cause degradation of the cell DNA and induce chromosome abnormalities. This activity could be one cause of the aneuploidization of cells that is often correlated with their emergence from diploid cultures as continuously growing cell lines.

It seems possible that cell-dependent mycoplasmas could exist and be present in tissue cultures. Such organisms would not be detected by the usual culturing procedures but would probably be capable of exerting some of the undesirable effects described in this review.

Mycoplasmas show such great biochemical diversity that there is little doubt that new effects of their impingement on the physiology of cultured cells will continue to be described, and past errors in interpretation revealed.

I wish to thank Professor M. G. P. Stoker for his advice and criticism.
REFERENCES


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Fig. 1. Polyoma-transformed BHK 21 cells infected with mycoplasmas and stained by the method of Marmion & Goodburn (1961). Mycoplasmas can be seen attached to the cell surface.

Fig. 2. Cells of the BHK 21 line after the hypotonic treatment and air-drying procedures used for the demonstration of chromosomes. Mycoplasmas are easily detected on the extended cytoplasm. Giemsa stain.
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(Facing p. 168)