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A REVIEW OF CELL CULTURE CONTAMINATIONS

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It is apparent that most laboratories employing tissue and cell cultures for experimental or diagnostic purposes encounter contaminations. If not detected and properly controlled or eliminated, contaminants may severely affect such investigations, and be the cause of incorrect interpretations. The Committee on Contaminations in Cells and Tissue Cultures of the Tissue Culture Association considered it as one of its functions to review the most pertinent literature related to contaminations with cells, bacteria, yeasts, molds, parasites, *Mycoplasma*, and viruses. The present review article is not intended to cover the subject completely but will, hopefully, be helpful to workers in the area of cell and tissue cultures who are concerned about the problem of contamination. Apparently many are not thoroughly familiar with the proper procedures for prevention, detection, and, perhaps, elimination of contaminants from the experimental systems that they employ.

CONTAMINATION IN CELL AND TISSUE CULTURES BY CELLS

The problems related to interspecific contamination of cell cultures have been known for some time (1–6). Immunological and karyological techniques have been widely used to detect such mishaps. Interspecific cell contamination was well illustrated in a recent publication (7). A line of cells designated guinea pig spleen (GPS) originated at Fort Detrick and had been disseminated to a number of laboratories. A combination of serological and karyological data revealed that the line actually consisted of L-M mouse cells and not GPS cells.

For a long time many investigators suspected similar intraspecific contaminations among human cell cultures. In 1966, tools became available to study the extent of such contamination (8). It was reported that 18 established human cell lines derived independently from Caucasian and Negro subjects contained the A isoenzyme of glucose-6-phosphate dehydrogenase (G-6-PD) and type 1 isoenzyme of phosphoglucomutase (PGM). The A isoenzyme of G-6-PD is found in 30% of the American Negro population, but not in Caucasians, while the type 1 PGM isoenzyme is found in 65% of both Negro and Caucasian populations. Since HeLa was the first human epithelial cell line established, and since it was derived from a Negro, it was concluded that most likely the other 17 established cell lines were HeLa cell contaminants.

In a follow-up study (9), identical findings were reported for 20 heteroploid human cell lines established between 1952 and 1957. Conversely, recently isolated diploid or near diploid human cell lines, as well as 100 primary tumor cultures, contained isoenzyme patterns consistent with their racial phenotype. Furthermore, *Mycoplasma*-contaminated, SV40-inoculated, X-irradiated, or other treated cultures did not alter their G-6-PD or PGM isoenzyme patterns.

These findings have been confirmed and extended (10, 11) using the G-6-PD isoenzyme types of human cell lines in sucrose-agar gel electrophoretic media as well as in cellulose acetate strips. The A and B types of G-6-PD were found to occur in cultured human diploid cell populations in accordance with racial origin of the donor, while the heteroploid cell lines established during the 1950's possessed A type G-6-PD irrespective of donor race. As has been pointed out (10), one should not conclude that because heteroploid cell lines were contaminated by HeLa cells all observed spontaneous "transformations" of human cells in vitro were due to contamination by HeLa cells. Such HeLa contaminations may have taken place after the spontaneous "transformation" had been ob-

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served. This is one possible explanation of the recently reported results (12) on a 4-year-old established cell line MA 160 which originated from a spontaneous "transformation" of adult human prostatic epithelium. It was found (11) that the MA 160 had the rapidly migrating A component of G-6-PD, even though it arose from a Caucasian male and still retained the Y chromosome. Other possible explanations presented (12) were that the patient had Negro ancestry or the A band of G-6-PD may eventually be found in all cells carried for long term in vitro. Work now in progress in several laboratories (13) using these newer techniques to monitor cell lines closely over extended periods of subculture will, hopefully, provide answers to these puzzling questions.

This information on inter- and intraspecific cell contaminations has made many investigators aware of the hazards of handling more than one cell line at a time in the laboratory. Consequently, as evidenced by the sparsity of reports in the literature on contamination of cells by cells, one may perhaps conclude that an increased awareness of the possibility of such contamination has in itself decreased the incidence. However, serological and karyological tests, as well as enzymatic assays of dehydrogenases, kinases, transferases, and esterases, should be performed on cell lines at regular intervals in order to monitor for this type of contamination.

Contamination by Bacteria, Yeasts, and Molds

Bacterial contamination occurs occasionally in most laboratories in spite of the use of antibiotics. In the absence of antibiotics, this type of contamination can usually be observed by the turbidity of the culture medium and, possibly, destructive effects on the cells. Whereas antibiotics kill many contaminants, their presence may only suppress others and permit the existence of a chronic or latent infection which is not readily apparent (14). Low order bacterial contamination (100 to 1000 bacteria per ml of tissue culture media fluid) in 20 of 73 cell cultures tested (27%) from 22 laboratories has been reported (15). The bacteria were identified as members of *Corynebacterium*, *Micrococcus*, *Bacillus*, and gram-negative enteric rods. There was neither turbidity of the cell culture media nor apparent cytopathic effect on the cells. None of the investigators knew about the bacterial contamination. The cultures generally had been checked only with thioglycolate broth media, without regard to the inhibitory activity of the antibiotic or the need for special nutrients after being subjected to antibiotic insult.

Pseudomonas aeruginosa can produce plaques in cell monolayers which are indistinguishable from viral plaques (16). Other strains of *P. aeruginosa*, *Pseudomonas fluorescens*, and *Alcaligenes fecalis* formed similar plaques on cell monolayers maintained in antibiotic-free medium, whereas strains of other bacteria like *Escherichia coli* and *Staphylococcus aureus* did not (17). The similarity of these bacterial plaques to viral lesions has also been demonstrated using an agar overlay technique.

The origin of bacterial contamination may be the original tissue or the medium employed, or may be related to faulty techniques. For the prevention of contamination, it appears to be advantageous to work in the absence of antibiotics. Although not always practical, it forces meticulous aseptic techniques, permits early recognition of contamination, permits use of antibiotics to treat infected cultures, and minimizes the development of L forms (14).

Yeasts, originating from many sources, are common contaminants of tissue cultures (14). Some grow slowly and are nontoxic; other yeasts grow best in the presence of living cells and fail to grow in aerobic blood agar plates (14). They can usually be detected by direct inspection, which is sometimes facilitated by removal of inhibiting antibiotics like mycostatin or fungizone from the cell culture media. Subculture in Sabouraud dextrose agar may be necessary. Treatment of cultures with mycostatin or fungizone is occasionally successful.

Infection with molds may be the result of air-borne contamination or human contact. High humidity increases the risk for contamination. Cold rooms appear to represent a special danger for bottle neck contamination of media containers. Molds tend to grow slowly and do not cause a serious problem for many short term experiments (14). Since Petri dish cultures are much more susceptible to contamination, it is advisable to avoid such cultures for long term experiments, and to carry continuous cell lines in closed culture containers. Microscopic inspection of cell cultures and inoculation into Sabouraud dextrose agar are the methods of detection. Treatment of cultures with mycostatin

or fungizone is sometimes successful. Usually it is necessary to discard contaminated specimens, taking care not to open them in the laboratory, and making certain that they are autoclaved before being washed (14).

The severity of the problem with these types of contaminants is illustrated by the fact that 18% of the cell lines submitted to one of the cooperating laboratories of the Cell Culture Collection Committee during 1968 were contaminated with various microorganisms (18), and 27% of the lines submitted in 1969 were similarly contaminated (19). It has been estimated that approximately 50% of cell cultures in current use are probably contaminated (19).

The following procedures (19) have been recommended to prevent such infection of cultures. Careful aseptic technique should be followed in all laboratory work. All tissue culture personnel should be thoroughly familiar with aseptic techniques and understand the ubiquitous nature of microorganisms. To prevent air-borne contamination, high efficiency particulate air (HEPA) filters, which are 99.97% efficient in removal of particles having an average diameter of 0.3 μ, should be used with laminar or mass air flow in tissue culture transfer rooms. Furthermore, housecleaning procedures of proven effectiveness, rapid turnover of sterilized equipment and supplies, and adequate monitoring techniques to detect contamination should be routinely employed.

Special methods of disinfection have been considered in order to prevent the introduction of contamination through the serum supplement, which is necessary in connection with many cell culture media. Holmgren and Smith (20) considered ultraviolet light. They found, however, that the dose necessary to kill *Mycoplasma* altered some components in the serum pertinent for the stimulation of reproduction of L cells. Until the details of other methods with similar purpose (special ultraviolet treatment, inactivation with β-propiolactone) have been sufficiently tested, the claims that such methods have successfully killed bacterial and fungal contaminants (*Mycoplasma* and viruses as well) without affecting the growth-stimulating components cannot be fully accepted.

Contamination by Parasites

"Spontaneous" contamination with free living soil amoebae has been recognized and reported in only a few isolated cases: in freshly trypsinized monkey kidney cells (21, 22), Chang's liver cells (23), dog lymphosarcoma cells (LS No. 30) (24), and in HeLa cells (25). Two species of two genera of amoeba, *Hartmannella* and *Naegleria*, have also been found in cell cultures inoculated from pharyngeal swabs or other clinical material (26–28). In one of these studies, amoebae were isolated from a population of "normal" individuals under surveillance for respiratory disease viruses (27). The ease of isolation from the human throat in cells of many animal species in culture, including primary, diploid, and long term cell lines, attested to the efficacy of tissue culture as a growth medium for these species of amoebae. In cell cultures, amoebae produce cellular damage resembling viral cytopathic effects (23, 26, 28–38). This can lead to the belief that a bizarre virus might be involved.

Morphology and culture characteristics. Most of the amoebic strains isolated from tissue cultures belong to a relatively large group found free living in the soil and resembling the species first described by Castellani (39) and termed *Hartmannella castellanii*. A key for the identification of the species is available (40). In fresh preparations, the small amoebae (10 to 20 μ), with a sharp, well defined nucleus containing a large karyosome, display a prominent contractile vacuole (38, 40).

The cytoplasm is composed of an inner granular endoplasm and a clear ectoplasm. The karyosome is eosinophilic and has been mistaken for a cellular viral inclusion, especially since these protozoa in tissue culture resemble tissue cells, in particular mononuclear phagocytes, and have been confused with them (24, 41). They tend to be round but assume various shapes depending on the environment. In culture, they frequently appear pear-shaped with blunt pseudopodia on the rounded end. They are sluggishly motile at room temperature and at times several spinelike pseudopodia in constant motion can be seen on the opposite end. These pseudopodia are characteristic of the *Hartmannella* amoebae and are not found in the *Naegleria* species.

The cysts are somewhat flattened spheres (10 to 21 μ in diameter) with a double wall which is folded and wrinkled and typically polygonal in shape. At low power magnification, these resemble yeasts or dead cells (21, 41). The cysts are very resistant to temperature and will readily

vegetate after storage at different temperatures for up to 9 months (26).

In tissue cultures inoculated with *Hartmannella* sp., an unusual degeneration progresses to complete destruction in 24 hr to 10 days (21, 27). Early in incubation, they can sometimes be detected by low power microscopy, especially in the periphery of the cell sheet where the small trophozoites and cysts accumulate and adhere to glass surface. Fresh mounts prepared on slides with cover slips, and examined with phase optics at higher magnifications, will usually permit a presumptive diagnosis based on the motility, characteristic nucleus, and contractile vacuole. Staining of either trophozoites or cysts is variable but various fixatives and stains have been used successfully (21, 42, 43). Jahnes, Fullmer, and Li (21) found that very good staining of the cystic form could be obtained with the Bauer reaction (44) which stained the entire cyst red. With azure eosin, the karyosome and nuclear membrane of the trophozoite stained blue, and the nucleus faint blue. The cytoplasm stained a moderate irregular blue throughout because of numerous vacuoles and granules. Amoebae grow well on agar containing edible bacteria and in tissue culture in the absence of bacteria. Material suspected of containing amoebae is cultured on a 1.5% water-agar plate which has been seeded with a "lawn" of bacteria (45, 46); relatively clear areas appear early, rapidly enlarge to macroscopic size in 24 to 36 hr (41), and bear a superficial resemblance to lysis by bacteriophage (47). The clearing effect is the result of a simple phagocytic process by the amoebae and is not caused by a lytic enzyme (48). Details for detection by direct microscopic examination, by staining, and by culture can be found in the papers by Culbertson (41, 42).

Stability. Survival at low temperatures depends on the presence of the cystic form. Amoebae can be recovered from culture fluids which have been stored at −50°C or lower temperatures for various periods of time (21, 49). The vegetative forms are killed by exposure to 56°C for 15 min, whereas complete killing of mixed cultures containing trophozoites and cysts is accomplished at 56°C for 3 hr, but not at 56°C for 2 hr. The optimum temperature for growth shows variation with the strain or species, some showing better growth and cytopathology at 33°C, others at 37°C (40). Motility of the trophozoite form is greater at 37°C than at room temperature (42).

Drug sensitivity. The amoebae are resistant to penicillin and streptomycin. Sulfadiazine has been shown to be effective in treating experimental *Hartmannella* infections in mice. Relatively large doses of amphotericin B in combination with large doses of sulphadiazine in the diet had significant activity against the mouse infection with a *Naegleria* sp. isolated from human spinal fluid (50). Thus, the *Hartmannella* amoebae are sensitive to sulfadiazine, and the *Naegleria* respond to amphotericin B (51). Whether these antibiotics would be effective in ridding tissue cultures of the amoebae is not known. Casemore (25) incorporated 10 μg per ml of metronidazole in the culture medium because of its effectiveness against *Trichomonas vaginalis* and found complete suppression of amoebae.

Source of infection. Three possible sources of tissue culture cells have been implicated: (a) air-borne contamination; (b) contamination from the oropharynx of the tissue culturist; (c) outgrowth from a contaminated or infected host organ.

Since these organisms are free living soil organisms, the presence of cysts in the air is not surprising. Kingston and Warhurst (40) isolated in tissue culture or on agar plates 36 strains of amoeba, 32 of which were *Hartmannella* amoebae, from samples of the outside and inside air of a hospital, either through a slit sampler or on settle plates. They observed that the rate of settling (0.28 amoeba per m^2 per min) did not suggest that chance contamination by settling would occur frequently during laboratory manipulations. They found high values for the particle diameters (33 and 37 μ) from the settle plate counts as compared to the size of the cyst, 12 to 31 μ, and suggested that the cysts were associated with particles of soil. They stated that air-borne particles of this size would be impacted in the nose. This may explain the relatively high recovery of amoebae from the nasopharynx of a presumably "normal" population as reported by Wang and Feldman (27). The relatively rare amoeba contamination of primary and established tissue cell cultures reported to date could derive from either source, air-borne or the human oropharynx. Casemore (25), reporting from a laboratory where the layout seemed to favor contamination from the environment, found that cysts kept at different temperatures for 9 months vegetated readily when placed in suitable me-

dium. Therefore, air-borne cysts could be a source.

Although Culbertson believes that contamination of tissue cell lines is more likely attributable to air-borne contamination, he has found amoebic lesions in the kidney of infected mice. If such a kidney were cultured, amoebae would grow into the culture. He and collaborators observed in mouse experiments following infection with the low virulent strains that the amoebae persisted in the trophozoite, precystic, and cystic stages for periods up to 1 year. It might be expected that cultures from animal organs may occasionally contain the *Hartmannella* amoebae deriving from the host (51).

Methods of detection.

1. Staining of tissue culture cells grown on cover slips.

2. Examination with phase optics of wet preparations at both low and high magnification.

3. Culture on agar plates seeded with a "lawn" of *E. coli.*

In one study (38), an agent isolated in tissue culture was believed to be a virus (Ryan virus). Electron microscopy revealed a protozoal cell type in all specimens inoculated with the Ryan agent. The pathology noted in the cells resembled closely that described following *Mycoplasma* infection (52). Mycoplasmas have been shown to produce "nucleolar caps" (53), and to cause breakdown of deoxyribonucleic acid (DNA) and alteration in the species of ribonucleic acid (RNA) produced by the cells (54, 55).

These papers suggest above all the importance of alerting investigators to the possibility of contamination with an amoeba, particularly because of the close resemblance of the cysts to cytopathic effects caused by several viruses, and of the trophozoite form to macrophages. Microscopic observation at higher magnification should enable the presumptive identification of the trophozoite by motility and by characteristic nucleus and vacuole. Culturing on agar or liquid medium containing bacteria should confirm the diagnosis. The source may be either an aerial contaminant or amoebae present in the original tissue, particularly since these parasites encyst under adverse conditions. Droplet infection from the human nasopharynx cannot be ruled out as a possible source. For prevention, aseptic technique and care in methodology should tend to eliminate external sources. Preliminary studies on the use of chemotherapeutic agents to suppress infection in tissue culture mark the beginning of control measures.

Contamination by Mycoplasma

Robinson, Wichelhausen, and Roizman (56) reported on contamination of human cell cultures with pleuropneumonia-like organisms (PPLO) in 1956. They cautioned that "the undetected presence of PPLO may invalidate or confuse observations made on presumably uncontaminated cultures." Since then the warning has been repeatedly stated and knowledge of the incidence and serious consequences of *Mycoplasma* infection has greatly increased (57–63). The presence of mycoplasmas has been shown to cause a wide variety of cytological, immunological, genetic, biochemical, and other changes. The insidious nature of the contamination, which does not necessarily change the gross morphology or growth, emphasizes the necessity for routine testing procedures and strict control measures. However, the etiology of infections may still be uncertain.

Effects of mycoplasmal infection on cell cultures. Gross macroscopic changes in tissue cells infected with *Mycoplasma* sp. can range from inapparent or minimal unsuspected alterations to cytopathology and cell destruction reminiscent of viral infections. Macroscopic changes in morphology can be so minimal that they are not suspected even though a culture can reveal high titers of *Mycoplasma.* Cytopathic changes may be related to depletion of arginine in the medium by the *Mycoplasma* (64, 65). Arginine is an essential metabolite for growth of some mycoplasmas (66, 67) and for mammalian cells (68, 69); it is used in the pathway to form energy-rich phosphate bonds (adenosine triphosphate (ATP)) (66, 67). Adenovirus (70, 71) requires arginine for maturation and does not replicate in *Mycoplasma*-infected tissue cells which are depleted of arginine. Type A influenza virus (72) and vaccinia virus (73) are also arginine-requiring. Not all cytopathology can be attributed to the arginine effect. Infection of human diploid and chick embryo fibroblasts with *M. orale* inhibited the growth of Rous sarcoma virus and the production of avian leukosis CF antigens (74). Arginine did not reverse the suppression of viral multiplication, since even lower levels than those reported effective by others reduced the amount of avian leukosis CF antigen formed.

Effects of cytopathic *Mycoplasma* sp. have been confused with virus infections. One cytopathic strain of *Mycoplasma* (75) caused an unusual degree of acidity and eventual degeneration of the tissue cells. Moreover, HEp-2 cells contaminated with this *Mycoplasma* inhibited the growth of measles virus. This strain of *Mycoplasma* was later identified as *M. hyorhinis* (76), a porcine strain. It is to be noted that, even with the acid-inducing mycoplasmas, frequent medium changes or passage of cell cultures may depress the cytopathology and cell destruction to an inapparent level (75).

Another type of cytolytic *Mycoplasma* was reported by Kraemer (77). In this case, the lytic effect was definitely prevented if enough L-arginine was added to the medium. One isolate, which was serologically identical with another isolate, was later identified as *M. hominis* type 1, yet three other *M. hominis* type 1 strains proved to be nonlytic. This demonstrated the possibility of differences within the antigenic species of *Mycoplasma.*

These and other studies of cytopathic *Mycoplasma* strains have emphasized the risk involved in attributing all cytopathic effects to viruses. Many properties are shared by *Mycoplasma* and viruses: (a) filtrability: a number of strains of *Mycoplasma* (78) produced cells capable of passing through a 0.22 μ Millipore filter, and it has been found (79) that the size of the smallest filtrable unit varies under different conditions of culture; (b) electron microscopic morphology: certain pleomorphic forms of rickettsiae, ornithosis virus, and *Mycoplasma* can be confused with one another (80); (c) sensitivity to ether (75); (d) ability to hemagglutinate (81, 82); (e) ability to cause hemadsorption (83); (f) resistance to some antibiotics; (g) inhibition of growth by antiserum (84, 85); and (h) induction of chromosomal aberrations (86, 87).

Most important are the effects of mycoplasmas on the macromolecular DNA, RNA, and protein synthesis, and on the genetics of the mammalian cell. Chromosomal aberrations and changes in the chromosome numbers caused by *Mycoplasma* sp. have been demonstrated in an established human amnion cell line (FL) (86). Some of these changes were irreversible on elimination of the mycoplasmas from the culture (87). Chromosome changes were also observed in *Mycoplasma*-infected human diploid cell lines (88, 89). The reduction in chromosome numbers in *Mycoplasma*-modified FL cells could not be explained by a loss of DNA, but may be interpreted by a rearrangement of the genetic material (90). Instability of DNA as detected by incorporation of ^{3}H- or ^{14}C-thymidine into the DNA of *Mycoplasma*-infected mouse cells, and its subsequent release into the medium as acid-soluble radioactivity, has been shown (91). In autoradiographs, the isotope in infected cells was distributed along the cell margins and was absent over the nucleus, whereas in uninfected cells the label was found over the nucleus (92). In other studies (93, 94) the radioactive label was also concentrated over the cytoplasm and on the surface of the mammalian cells, and a new species of RNA with a sedimentation constant of 14 to 20 S was found in cultured cells contaminated with *M. hyorhinis.* Similar results were obtained with *M. pulmonis* and *M. neurolyticum.* In infections with *M. arthriditis, M. fermentans, M. hominis, M. orale* I and II, and *M. pneumoniae,* the temporary alteration in RNA proved reversible. The presence of two ^{32}P-labeled RNA fractions has been demonstrated in many *Mycoplasma*-contaminated cell cultures (95). These fractions are unusual for mammalian cells and correspond to RNA components of procaryotic cells. Exposure of tissue cells to a variety of agents, carcinogens, antibiotics (actinomycin, mitomycin C), alkaloids, aflatoxin, infection with *Mycoplasma,* polyoma, and Herpesvirus, and ultraviolet and ionizing radiations cause "nucleolar caps," a term describing nucleoli whose components have segregated into zones (53). It is suggested that the common denominator may be interference with DNA synthesis or inhibition of DNA-dependent RNA synthesis. A competitive effect for nucleic acid precursors, which may interfere with the host cell DNA synthesis, is suggested as an explanation for the chromosome aberrations observed after mycoplasmal infection of diploid human cells (96).

In addition to the previously described effects on virus infections, the presence of mycoplasmas in other systems has inhibited (97, 98) or enhanced (99) the propagation of viruses and has been implicated in possible carcinogenic effects in neoplasia. Mycoplasmas can affect virus-induced transformation of cultured human cells (100). They are also responsible for chromosome changes in such systems (101). A relation between mycoplasmal infection and an irreversible reduction of the tumor-producing capacity of human cell lines has been demonstrated (102).

Detection of mycoplasmal contamination. There is a number of methods for the detection of mycoplasmal contamination. These include growth on agar and in broth and the examination of cells by staining methods, immunofluorescence techniques, and electron microscopy. In addition, several biochemical methods have been proposed.

For demonstration of the characteristic growth on agar (97, 103), the medium on which *M. pneumoniae* was first grown successfully (104) has been used by many laboratories. Hayflick has described the complete formulation and method of preparation (103). *Mycoplasma* are fastidious in their requirements for media of special composition, and it is unlikely that present culture media contain all of the essentials for growth of all species of *Mycoplasma*. There is also evidence of variations in the ability of different laboratories to culture mycoplasmas (94). Certain strains of *Mycoplasma* require tissue culture for the initial propagation following which culture on *Mycoplasma* agar and broth media can then be obtained. It has been suggested (105) either that the tissue culture has a greater sensitivity in meeting the initial nutrient requirements of certain strains of *Mycoplasma* or that some species may require a living cell for maturation.

In addition to colonial morphology, preliminary presumptive identification depends on the characteristic growth of the central part of the colony into the agar. If stroked with a bacteriological loop, part of the *Mycoplasma* colony remains embedded, whereas a bacterial colony will rub off. The centers of mycoplasmal colonies are stained a deep blue, with a lighter blue periphery when treated with the Dienes stain (106). One formula for the Dienes stain is as follows: 2.5 g of methylene blue, 1.25 g of azure II, 10 g of maltose, 0.25 g of sodium carbonate, 100 ml of distilled water. By means of a cotton applicator, a thin film of stain is applied to cover slips. Blocks of agar, 6 to 8 mm, containing the suspected colonies, are cut out aseptically and placed colony side up on a glass slide. The dye-coated cover slips are then gently placed, stain side down, on the agar blocks so that a projecting rim of cover slip extends on all sides of the agar block. Paraffin containing 10% Vaseline can be used to seal the mounts to prevent drying. Staining is complete in a few minutes. In such preparations, colonies of mycoplasmas stain a dense bright blue in the center with a lighter periphery. Young viable bacterial colonies are also stained, but are decolorized in about 30 min. Mycoplasmal colonies retain the stain.

The presence of pseudocolonies on the surface of the agar can be confusing (107). They are composed of calcium and magnesium soaps which form crystalline structures on the agar surface, closely mimicking the colonial morphology of mycoplasmas. One method for identification of the species is growth inhibition with specific antisera (108).

The colonies should not revert to bacterial forms when grown in the absence of antibiotics. Such a reversion would indicate that the original colonies were L forms.

Various staining methods are used for the demonstration of mycoplasmas directly on mammalian cells. An intensified Giemsa stain permits the visualization (109) of pink coccobacillary forms on the surface of the bronchial epithelial cells in sections from animals infected with *M. pneumoniae*. The May-Grünwald-Giemsa stain has been used to demonstrate *M. pneumoniae* in human cell culture (110). Mycoplasmas can be detected with great ease in cells viewed under phase optics after exposure to hypotonic treatment, air-drying, fixation, and staining with orcein (111, 112). They are primarily located in the extended cytoplasm of the cells and in the intracellular spaces. It is recommended that *Mycoplasma*-free indicator cells (FL cells) be employed for inoculation of culture supernatants to be tested. Complete agreement between this method and agar growth methods has been reported.

Detection of mycoplasmas in mammalian cells has been accomplished by the use of immunofluorescence techniques (113). A polyvalent antiserum is necessary (114). Although obviously not the most practical routine method, mycoplasmal infection of cultured cells can be detected with the electron microscope (38, 61).

One biochemical method is based on the presence of arginine deiminase activity (15). It was later found (67) that only some strains of *Mycoplasma* possess this activity, upon which the test depends, and that a fairly high titer of *Mycoplasma* was necessary. Furthermore, although arginine deiminase activity is not found in animal cells, some bacteria possess this enzyme and can give a false reaction with respect to mycoplasmas. Another biochemical method is

based upon thymidine cleavage (115). The demonstration of two new RNA fractions in *Mycoplasma*-infected cells by agar gel electrophoresis has also been proposed as a diagnostic method (95).

Sources of contamination. Much speculation and experimentation during the last decade have not conclusively established the source(s) of mycoplasmal contamination. Since it is firmly established that primary cell cultures are seldom contaminated, whereas continuous cell lines are frequently contaminated (116), the tissue of origin is not the likely source, but some of the steps involved in the handling of the cultures in the laboratory must be at fault. The conversion of ordinary bacterial contaminants by the presence of antibiotics into bacterial L forms (resembling or indistinguishable from mycoplasmas) has been considered a likely possibility by many investigators (20, 58, 59, 97, 103, 117). Carski and Shepard (63), however, were unable to culture *Mycoplasma* from cultures to which had been added B forms of a number of antibiotic-sensitive bacteria. The obvious possibility that either the serum supplement (20, 58, 62, 103, 116, 118, 119), embryo extract (103), or trypsin (116) may contain the microorganisms has been considered, but until recently no real evidence has been obtained. *M. arginini* and several unclassified mycoplasmas have now been isolated from a substantial amount of commercially available lots of fetal bovine serum when large volumes of serum were inoculated into broth medium (120). Penicillin (suspected of containing filtrable L forms) has so far not proven to be a source (103). Careless techniques are presumably, to a great extent, responsible for the introduction of the contaminants into the cultures or reagents. Mycoplasmas can be disseminated from the oropharynx via droplets, saliva, mouth pipetting, or the contaminated fingers of technicians, as has been proposed by several investigators (63, 103, 116, 118). Careless technique has been fostered by the use of antibiotics which keep down bacterial contaminants but may selectively permit mycoplasmas to persist. It is interesting in this connection that the incidence of *Mycoplasma* contamination was related to the nature of the laboratory (121). Tissue cell-producing laboratories, and those using cells for propagation of microorganisms, had the highest contamination incidence. However, laboratories which used cells for metabolic studies were free of mycoplasmas in this study. Exchanges of cell lines between laboratories (62) and cultures distributed from commercial suppliers, if contaminated, represent an obvious source for the introduction of mycoplasmas into a laboratory. Also, where tissue culture laboratories and animal colonies are in close proximity, it is conceivable that the microorganisms may be transferred from infected animals to the cultures (119).

Prevention procedures. Rather simple methods of prevention of mycoplasma contamination are available. Among the recommended procedures are: rigid aseptic techniques, including avoidance of the use of mouth pipetting; less widespread use of antibiotics, stock cultures being maintained without antibiotics to enforce more rigid techniques; separate feeding and culture of each cell line. Frequent checking of cultures and media for contamination is important. As an alternative to eliminating antibiotics from the cultures, the use of antibiotics known to be effective against mycoplasmas in doses nontoxic to cells may be preventive. These methods have been discussed in several publications (56, 59, 62, 63, 103, 112). Careful consideration is needed to avoid contamination being carried to the cultures from animal colonies (119). Filtration of tissue culture media and solutions may not completely eliminate mycoplasmas. Sterilization of serum with ultraviolet light may affect growth-supporting components needed by the mammalian cells (20), and heat inactivation (30 to 60 min at 55°C) is not a certain safeguard (57). In many laboratories noninfected stock cell lines are stored in liquid nitrogen for replacement of cell lines which become infected. One report describes the prevention of mycoplasmal contamination during many years by refiltration of all commercially obtained media and sera followed by 10 days' storage at refrigeration temperature, and the employment of rigid (but feasible) aseptic techniques combined with frequent culture tests. By these procedures contamination is avoided in spite of a sizable experimental *Mycoplasma* program carried on by the same technicians within the laboratory (112).

Methods for elimination of mycoplasmas from tissue cultures. Various methods for elimination of mycoplasmal infection have been reported. Most of these depend on the sensitivity of the mycoplasmas to antibiotics such as aureomycin (59), kanamycin (60, 122), novobiocin (123), or tylosin (146). Antibiotic treatment has been combined

with heat treatment (124), a short term exposure to hypotonic conditions (125), or a change to different nutritional conditions (112). There are reasons to exercise caution in the determination of the potency of antibiotics against mycoplasmas by methods employing acellular media alone (126). Usually a higher dosage than is indicated by the minimal lethal dose found in broth for the isolated *Mycoplasma* is found to be necessary to clear the cell culture of mycoplasmal contamination (127). Heat treatment alone has worked under certain conditions (128), but has not been generally successful (129). Short term exposure to antiserum specific for the contaminant has eliminated mycoplasmas in the hands of some investigators (129, 130). Complement addition may be necessary.

There apparently are factors which bring about a variable response to treatment. Among them is the development of resistance to various antibiotics.

Virus Contamination

For many years there has been a general suspicion that undetected viruses might be present in many cells cultured for experimental work (14). With today's knowledge (much of which was presented at the Conference on Cell Cultures for Virus Vaccine Production of the National Cancer Institute in December 1968) there are reasons to be concerned about this problem. As with other types of contamination, the sources include the tissue from which cells are cultured, serum, reagents, the technician, and the environment. The tissue appears to represent the most important source to consider.

Tissue as potential source of viral contaminants. Viral contaminants can be agents which (a) show high incidence of infection in the host; (b) produce a systemic infection and disseminate to tissues commonly used in cell culture, or have a specific tropism for such tissues; (c) remain in the tissues of the host for a sufficiently long time to make isolation likely; (d) produce silent or at most a mild disease; and (e) will replicate or at least survive under in vitro cell culture conditions (131). Recent knowledge about helper and satellite viruses complicates the exact definition of potential viral contaminants.

Human viruses. It is generally believed that there is a rather limited occurrence of contaminating viruses in human tissues (132). Melnick (133) has listed the known latent viral infections of man as follows: herpes simplex infection and other members of the Herpesvirus group (varicella virus, herpes zoster virus, and cytomegalovirus); latent adenovirus infections (common); a new Herpes type virus, apparently widespread in human beings (cultured from Burkitt lymphoma cells). Among other latent infections are measles virus associated with subacute inclusion body encephalitis and, particularly, congenital rubella. Other viruses are the satellites of adenovirus, whose growth depends on a late state in the replication of the helper adenovirus. To these must be added viruses recovered, generally as a result of intrauterine infection, from the fetus, stillbirth, newborns, and malformations: rubeola, vaccinia-variola, poliovirus, Coxsackie virus, ECHO virus, mumps, influenza virus, western encephalitis, lymphocytic choriomeningitis, rabies, infectious hepatitis, and psittacosis (132).

Monkey viruses. Monkey cells may contain many virus contaminants. Simian virus 40 (SV40), a tumor-producing virus when inoculated into newborn hamsters, was first isolated from cultures of Rhesus or Cynomolgus cells. It is not known to be oncogenic in vitro or in vivo to the cells of the species in which it is indigenous. It produces a cytopathic effect in African green monkey cells and transforms hamster, mouse, monkey, and human cells. It is found frequently as a contaminant of kidney preparations of various nonhuman primates (Rhesus, Cynomolgus, and possibly Patas monkeys) (132). The incidence of infection in Rhesus monkeys has been up to 100%. Other viruses which have been found repeatedly in monkey kidney cultures are herpes B virus (herpesvirus simiae), paramyxovirus SV5, and foamy virus (133), but over 50 viruses have been isolated from several nonhuman primate materials, mostly from kidney cell cultures, but also some from throat swabs and fecal extracts. Adenoviruses predominate, especially as recovered from tissue culture preparations. Of greatest concern is the herpes B virus, which is present in a variety of simians, primarily in Asian species of monkeys, and is fatal for humans. Only mild or inapparent symptoms occur as a result of infection in the monkey (132).

Bovine viruses. Bovine adenoviruses, bovine herpes virus 1, parainfluenza 3 virus, certain enteroviruses, and bovine diarrhea virus are potential contaminants of cell cultures. Herpesvirus 1, parainfluenza 3, the enteroviruses, and

bovine diarrhea virus are especially significant, since they may be present in both fetal and adult animal tissues (132). Infectious bovine rhinotracheitis virus has been isolated from samples of commercial fetal calf serum (134), and bovine diarrhea virus from bovine serum to be used in tissue culture (135).

Chicken viruses. Several chicken viruses are potential contaminants of cell cultures: avian adenoviruses, leukosis viruses, avian encephalomyelitis virus, avian infectious bronchitis virus, and Newcastle disease virus. Avian adenoviruses and lymphoid leukosis viruses appear to be the most likely candidates. The other viruses may be significant if the flock is in the active stages of infection (136).

Most flocks of chickens have been found to be infected with leukosis viruses, which are usually introduced early in life by contaminated respiratory vaccines (133). The virus is transmitted horizontally through saliva and feces; vertical transmission has been demonstrated from the viremic hen. Leukosis virus acts as a helper for Rous sarcoma virus, producing the protein coats. It also interferes with Rous sarcoma virus. There is recent evidence of a Herpes-like virus being associated with Marek's disease. The virus grows in chick cell cultures, and should be considered as a potential contaminant of chicken embryo cell cultures and a likely contaminant of cultures prepared from adult tissue (137). Avian encephalomyelitis virus has been described in cultures of chicken embryo cells without cytopathic effect. Infectious bronchitis virus has not been reported as a contaminant, but has the necessary characteristics. Newcastle disease virus has been present as a contaminant of chick embryo fibroblast cultures.

Duck viruses. The obvious possibilities include duck plague and eastern encephalitis, but they have not been firmly established as contaminants. Duck hepatitis could be considered as a possible candidate (136).

Dog viruses. Eleven viruses have been isolated from the dog (138). The viruses associated with canine hepatitis and Herpesviruses appear to be of special importance (139). Canine distemper and infectious canine hepatitis occur as common infections during the 1st year of life. This may also be the case with parainfluenza and canine Herpesvirus (138). The canine lymphosarcoma is of frequent occurrence (133).

Rabbit viruses. Many viruses associated with various genera of rabbits and hares are known (140). However, isolation of contaminating viruses appears to be rare. Enders has isolated a rabbit Herpesvirus (141). Six viruses appear to infect only wild species under natural conditions. Several viral agents may afflict domestic or laboratory rabbits; myxoma and rabbitpox viruses produce fulminating infections with high mortality, and oral papilloma viruses, virus III, and possibly reoviruses produce only minor or inapparent infections.

Satellite and helper viruses. Satellite viruses are morphologically and serologically unrelated to the viruses with which they may be found. They may grow independently of the companion virus, or (for example, those of adenoviruses) they cannot replicate alone and remain undetected unless a replicating adenovirus is present. Replication of the associated satellite virus interferes with the replication of the companion virus.

Helper viruses are usually not found with the virus that they help under natural conditions. An exception is the Rous-associated virus-Rous sarcoma helper system. The Rous-associated virus is needed to form the viral capsid. Newcastle disease virus needs the helper parainfluenza virus type 3 in calf kidney cells, and human adenoviruses undergo an abortive infection in simian cells and need SV40 as a helper virus. A step in the transcription or translation of late events in the cycle of the virus is apparently not performed efficiently in the simian cells in the absence of helper viruses (142).

Omnipresence of viruses. Huebner and Todaro (143) have presented evidence that cells of many, and perhaps all, vertebrates contain information for producing C type RNA viruses. According to their theory, carcinogens, irradiation, many and frequent culture transfers, and the normal aging process may all favor the partial or complete activation of these genes. This activation may result in many different forms of cancer.

Serum as a potential source of viral contaminants. Molander and collaborators (144) examined commercial sera and specially procured batches of fetal calf serum for possible viral contamination. One out of 13 samples of the commercial serum had evidence of cytopathic effect in bovine tracheal cells. In a following report the same group (134) found that 14 out of 148 lots of bovine serum showed evidence of cytopathic or noncytopathic viruses. The noncytopathic vi-

ruses were demonstrated by interference effects. Two isolates were identified as infectious bovine rhinotracheitis virus. There were four well documented incidences of bovine cell line contamination with bovine diarrhea virus through the medium with fetal bovine serum. This virus was revealed by fluorescent antibody tests, since there was no obvious cytopathology. The most recent data from this group show 18 viral isolates from 17 lots of serum out of 175 tested. The virus-positive lots include fetal calf, newborn calf, agamma newborn calf, and adult bovine serum. Viruses have also been isolated from equine serum (145).

Technician and environment as a potential source of viral contaminants. Although no literature of great significance to this problem appears to be available, the possibilities for contamination seem to be numerous.

Method for prevention and detection of viral contaminants. General precautionary laboratory procedures including rigid aseptic techniques (14, 112) are important to diminish the risk of contamination within the laboratory. Control measures relating to the tissue of origin depend, in part, upon proper pretesting procedures of the monkeys, dogs, flocks of chickens, etc. from which the tissues are obtained. The testing of the actual cultures for the presence of inapparent, persisting virus infection may involve complicated procedures which cannot be carried out routinely. Eight procedures for recognition of latent virus infections have been listed by Melnick (133):

1. Direct examination of cultured cells and tissue culture fluids in the electron microscope. Sensitivity of the technique can be increased by density gradient ultracentrifugation.

2. Examination of cultures for infectious virus by passage to susceptible cells.

3. Examination of cultures for interfering properties against known viruses, particularly in avian tissue cultures, against the transforming action of Rous sarcoma virus.

4. Fusion of test cells with susceptible cells to determine whether this leads to expression of infectious virus.

5. Analysis of the infective dose-response curve to see whether it fits single or double hit kinetics. This would determine whether the virus seed has become contaminated with another genome from a known or unknown source in the tissue culture system in which it was grown.

6. Addition of helper viruses to the test cultures to determine whether a new virus appears (satellite) or whether a double hit kinetic curve can be changed into a single hit curve (PARA- and MAC-adenoviruses, murine sarcoma-leukemia viruses).

7. Tests of the donor tissue for the presence of group-specific antigens (such as those of avian leukosis virus) with selected sera that do not react with coat protein antigens.

8. Homology studies to determine whether mRNA in the test cell hybridizes with nucleic acid of unknown viruses. This method has been applied for detecting mRNA of adenovirus and papovavirus in virus-induced tumors, and should also be applicable to cells carrying other incomplete viral genomes.

Additional methods include long term cultivation of the suspected cells and examination for hemadsorption.

Preventive measures to avoid virus contamination through the serum component in the medium include (a) testing and elimination of batches of sera and (b) methods for sterilization. Various methods for virus screening are being adapted by the commercial serum suppliers. Methods for sterilization have been considered for years. They include heat inactivation, filtration, centrifugation, exposure to ultraviolet light, X-rays, microwaves, and treatment with β-propiolactone. It appears that intensive testing of such procedures is necessary to evaluate their effectiveness in assuring sterility and their lack of destructive effects on the serum components necessary for optimal cell growth.

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