PLEUROPNEUMONIA-LIKE (MYCOPLASMA) INFECTIONS OF TISSUE CULTURE

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The frequent contamination of animal cell lines by pleuropneumonia-like organisms (PPLO) reported by Robinson, Wichelhausen, and Roizman (1956) and by Collier (1957) is of obvious consequence to research workers using tissue culture systems. The presence of these microorganisms could influence results obtained in diverse studies, for example, of nutritive requirements of the cells or of viral antigens grown in them. The cultural methods commonly employed for the demonstration of PPLO have been somewhat complicated, but they are not difficult with the medium of Barile, Yaguchi, and Eveland (1958).

To gain a clearer comprehension of the problem we have studied several aspects of the PPLO contamination of tissue cultures. An interesting model of host-parasite relationships emerges. Growth of PPLO occurs only in the presence of tissue culture cells, which are able to support growth of PPLO to high concentration without manifesting important cytopathic changes. It seems clear that unwanted PPLO infections of cell lines need occasion little future difficulties since it is possible to render the cell lines free of PPLO with antibiotic treatment.

Artificial media for the growth of PPLO from tissue cultures. A) The medium of Barile, Yaguchi, and Eveland (hereafter called medium I) consists of 3.7% commercial brain heart infusion, 0.2% powdered yeast extract, 1.3% agar, and 15% outdated human bank blood (which contains citrate-glucose solution). In 3 to 4 days this medium grows distinct colonies that may be easily counted and may be transferred by simple streaking with a bacteriological loop.

Modifications in the medium were tested by plating out 0.01 ml of 10-fold dilutions of tissue culture fluid of a HeLa cell line, and observations were made daily of the size and number of colonies. Usually 1 to 20 colonies were seen with the 10^{-4} dilution. This corresponds to 1×10^6 to 2×10^7 PPLO/ml. Variations in pH between 6.8 and 8.2 before addition of the blood were without effect. Incubation in an atmosphere of 5% CO_2 did not improve the result. Elimination of the yeast extract decreased the size of the colonies somewhat. The agar concentration is about optimal, in that lower concentrations are inconveniently soft.

Some of the substances reported to support protoplasts (Weibull, 1958) were added to the medium. Sucrose (10%) was without effect, and at 17% (0.5 M) it prevented growth. $MgSO_4$ at 8×10^{-3} M had no effect, and at 2.5×10^{-2} M it decreased growth. Penicillin (100 units/ml) did not alter growth.

B) A medium consisting of 15% human bank blood in commercial blood agar base (hereafter called medium II) gave consistently better growth than medium I and was somewhat easier to prepare.

C) During the course of attempts to substitute 10% human serum for blood in medium II the "satellite" phenomenon was observed in the vicinity of an incidental white micrococcus colony. Elsewhere in the plate, inoculated heavily by pouring, the colonies were very small but they increased markedly in size near the coccus colony. The effect of growth factors for Haemophilus was then studied with yeast extract and horse blood extract (Levinthal) prepared according to Alexander (1958). With these a satisfactory transparent medium could be prepared with human serum. The most favorable proportions were 10% yeast extract, 10% Levinthal extract, and 10% human serum in blood agar base (hereafter designated medium III), and with this medium, colony growth was equivalent or somewhat better than on medium II. The advantages of transparent medium for the observation of the microscopic morphology of PPLO colonies are well known. If in medium III the human serum was replaced with bovine

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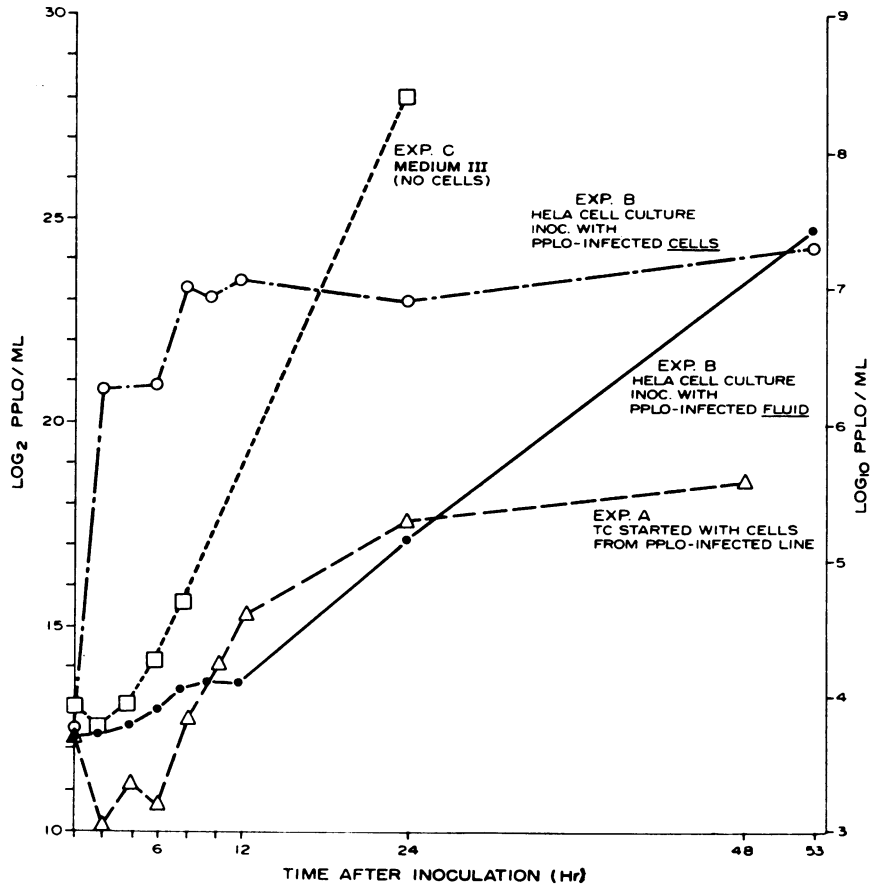

Fig. 1. Growth curve of PPLO in fluid phase of HeLa cell cultures and in artificial medium

fetal serum, or bovine plasma albumin (fraction V), growth of PPLO was markedly reduced. Adsorption of the human serum with 4 g type A zymosan/ml to remove properdin did not affect the result.

Although the observation of the satellite phenomenon and the growth-promoting effect of blood and yeast extracts suggested relationships of the PPLO to Haemophilus, other observations did not accord with this idea. Thus, in the absence of Levinthal stock, 5 μg hemin/ml had little or no growth-promoting activity, and in the absence of both Levinthal stock and yeast extract 0.1 μg diphosphopyridine nucleotide (DPN)/ml had no effect. The usual procedure of inactivating DPN by autoclaving could not be employed because this treatment rendered the Levinthal stock so inhibitory for PPLO that no colonies developed.

A liquid medium in which agar was omitted from the formula of medium III supported rapid growth of PPLO with a generation time of 1.3 hr (Fig. 1). This is at least twice as fast as the 3.2 ± 0.8 hr by Keller and Morton (1954) for culture of human genitourinary origin in "PPLO" medium (the data of their Fig. 3 appear to give 2.2 to 2.8 hr). It is about the same as the 1.1 hr reported by Laidlaw and Elford (1936) for saprophytic mycoplasma of sewage origin in Hartley's digest broth containing Filde's extract, and the 1.3 hr reported recently by Butler and Knight (1960) for similar species in Edwards' medium.

Failure of tissue culture medium (without cells) to support growth of PPLO. The studies of growth requirements for PPLO colonies suggested that tissue culture medium by itself might not be adequate for the multiplication of these organisms. Solid media based on tissue culture formulas did not support the development of

TABLE 1

Incidence of PPLO contamination of cell lines maintained in Virus and Rickettsia Section

Laboratory	Type of Tissue Culture	PPLO Present
A	HeLa-calf serum line	+
	Rabbit kidney line 10/58	+
	Monkey kidney line 1/58	+
	Primary rabbit kidney	−
B	HeLa-calf serum line	+
	Monkey kidney line (Melnick)	+
	Primary hamster kidney	−
C	Human amnion line 185	+
	HEp-2	+
	HeLa-human serum line	−

colonies. This was noted with a medium in which the HeLa cell line had been maintained for years (40% human serum plus 60% Hanks' balanced salt solution), and with the medium used to maintain the HeLa cell line in this laboratory (20% human serum plus 10% bovine fetal serum plus 70% balanced salt solution containing Eagle's amino acids and vitamins and 1 mmole arginine).

The latter medium as a liquid medium without agar did not support growth of PPLO either, although it maintained the viable count when incubated at 37 C overnight. The result was not significantly different when the medium had been "conditioned" by remaining in contact with a monolayer of HeLa cells for 3 days.

Frequency of PPLO contamination of cell lines. All of the cell lines being maintained at the Virus and Rickettsia Section were examined for the presence of PPLO. To do this a suspension of cells was prepared by scraping the cell monolayers into the supernatant or by freezing and thawing a tissue culture, and 0.2 ml inoculated onto medium I. Transfers to new media were made once, or twice if necessary, to ensure that cell masses were not confused with PPLO colonies. Isolation of PPLO in each case fulfilled the following criteria: (i) small colonies with typical "fried egg" morphology, (ii) invasion of agar by colony rendering it difficult to dislodge, and (iii) lack of bacterial morphology in Giemsa stained preparations. The isolates were not culturally distinguishable from standard PPLO strains T-5 and C-2-7 received through the courtesy of George H. Rothblat and Harry E. Morton of the University of Pennsylvania. Seven of eight cell lines were contaminated, and neither of two primary tissue cultures (Table 1).

Frequency of PPLO contamination of virus and rickettsia strains. Forty-five virus and rickettsia strains were selected to represent various laboratories of the Virus and Rickettsia Section and to represent varied passage histories. Strains were sampled by obtaining a tube of the virus material as ordinarily kept by the laboratory for use in other studies. The technique was the same as that used for culture of PPLO from cell lines. The results are given in Table 2 according to the passage history. Of 26 strains with most recent passages in embryonated eggs and animals, all were free of PPLO. A strain of vaccinia, with a single passage on chorioallantoic membrane, after 3 passages in HeLa cells, was contaminated with PPLO. Of the strains from primary tissue cultures, the record was mixed. Of six strains with recent monkey kidney passage, all were free. One strain from hamster kidney was free; another, with an older history which included passage in human embryonic lung and monkey kidney tissue cultures, contained PPLO. All five strains of "foamy agent" from rabbit kidney were contaminated, although three of these had earlier histories in HeLa. It may be pertinent that the primary isolation of "foamy agent" strains required prolonged maintenance of monkey kidney tissue cultures (Carski, 1960). Of five strains from cell lines, four were contaminated with PPLO. An incidental finding was that five virus strains from mouse brains were contaminated with bacteria (B forms).

"Normal behavior" of PPLO in tissue cultures. The growth curves in Fig. 1 were determined by plating out 0.01 ml of 10-fold dilutions on medium II. The diluent was in all cases 40% human serum and 60% Hanks' balanced salt solution. In experiment A, a bottle of cells from a PPLO-containing line was transferred in a routine way, and the PPLO counted in the supernatant fluid at intervals (the 0-time reading represented the cell inoculum). To do this, an unselected bottle culture containing 10 million cells from another laboratory was washed three times in balanced salt solution, treated with 5 ml of 0.25 per cent trypsin, and 5 ml added of growth medium (20% human serum, 10% bovine fetal serum, 70% Eagle's amino acids and

TABLE 2

*Incidence of PPLO contamination of virus and rickettsial strains according to passage**

A. Found contaminated with PPLO

Latest Passage in:	Infectious Agent	Total
HeLa	3 Adeno, 1 herpes simplex	4
Hamster kidney	1 E-11 (Uppsala, only 1 passage in MK and human embryonic lung)	1
Rabbit kidney	5 Foamy agent (isolated in MK, 3 strains passed in HeLa)	5
Chorioallantoic membrane (CAM)	1 Vaccinia (isolated on CAM, passed 3 times in HeLa, then once on CAM)	1
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B. Found not contaminated with PPLO

Most Recently Passed in:	Infectious Agent	Total
HeLa	1 Coe	1
Allantoic fluid	4 Influenza, 1 mouse pneumonitis	5
Suckling mice	3 Coxsackie	3
Mouse brain	7 SLE, 1 YF, 1 CTF, 1 LCM	10
Chick embryo	1 EE, 1 WE, 2 JBE	4
Yolk sac	1 Psitt, 1 ET, 1 MT, 1 RP	4
Hamster kidney tissue culture	1 Uruma	2
Monkey kidney tissue culture	2 Influenza, 1 CA virus, 1 Coxsackie, 1 P 1, 1 E-7	6
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* Abbreviations:

Adeno = adenovirus
CA = croup-associated
CTF = Colorado tick fever
E-7 = enterovirus type 7
E-11 = enterovirus type 11
EE = eastern encephalitis
ET = epidemic typhus
HK = hamster kidney tissue culture
JBE = Japanese B encephalitis
LCM = lymphocytic choriomeningitis
MK = monkey kidney tissue culture
MT = murine typhus
P 1 = poliovirus type 1
Psitt = psittacosis
RP = rickettsialpox
SLE = St. Louis encephalitis
YF = yellow fever

vitamins in balanced salt solution, plus 50 μg streptomycin and 100 units penicillin per ml). Of this, 3.0 ml were diluted to 10.0 ml in growth medium and inoculated into a milk dilution bottle. The count of PPLO rose after 6 hr and reached a maximum of 5×10^5/ml at 48 hr. The curve was probably determined not only by the PPLO inoculated but also by the number of HeLa cells present to support growth of PPLO.

In experiment B of Fig. 1, the washed cells and the fluid of a culture of the PPLO infected HeLa line were inoculated separately into bottle cultures (containing about 15 million cells) of a HeLa cell line that had previously been rendered free of PPLO by treatment with tetracycline as described below. The cell inoculum was diluted to give a PPLO colony count about the same as the fluid inoculum. The growth curve from the cell inoculum rose to a high level in 2 hr, reflecting release of PPLO from the cell surface into the fluid (see below). The growth curve from the fluid inoculum rose more slowly after 12 hr to reach a similarly high level in 48 hr. The maximal rate of growth from the fluid inoculum corresponds to a generation time of about 3.5 hr. In curve C is shown the growth of PPLO in cell-free liquid medium III. The inoculum was again tissue culture fluid diluted to an appropriate level. The most rapid rate of growth in this medium was at a generation time of 1.35 hr between 6 and 8 hr, and 1.29 hr between 8 and 24 hr.

Continued release of PPLO from a contaminated HeLa cell monolayer is shown in the results of Table 3. The fluid of a bottle culture of a PPLO-infected line was removed, the cell layer and inside of the bottle washed three times with 10 ml of balanced salt solution, fresh growth medium added, and the bottle incubated at 37 C. At 1 hr and again at 2 hr the operation was repeated. The PPLO were counted in the tissue culture medium that had been in contact with the cells and also in the balanced salt solution of the third wash. There was only a slight reduction in the count of PPLO as a result of this procedure. The continued release presumably arises from the large reservoir of PPLO associated with the cells, as illustrated by the

TABLE 3

An attempt to remove PPLO from contaminated HeLa cell culture by repeated washing with balanced salt solution and incubation in fresh medium

	PPLO/ml	
	Tissue culture medium	Balanced salt solution of third washing
At start	3.6×10^6	2.0×10^4
Fresh medium added, cell culture incubated 1 hr	1.2×10^5	1.1×10^3
Fresh medium added, reincubate 1 hr	8.6×10^4	2.0×10^3
Fresh medium added, reincubate 1 hr	4.2×10^4	

fluorescent antibody studies described below, and by the electron microscope studies of Edwards and Fogh (1960).

Effect of antibiotics. The antibiotic sensitivity of PPLO of human origin has been studied by Robinson, Wichelhausen, and Brown (1952), who found PPLO to be sensitive to chlortetracycline, oxytetracycline, and chloramphenicol but resistant to penicillin and streptomycin. In the present study, the drugs listed in Table 4 were studied by means of sensitivity discs. Heavily streaked medium I was incubated 24 hr and commercial sensitivity discs (high concentration) were placed on the plates. Zones of inhibition were recorded after 5 to 7 days. If present, the zones were large and easily detected but their diameters were not recorded. Six strains of PPLO isolated from tissue culture and one (Alf.) isolated from a human case of nonspecific urethritis were tested. No differences in antibiotic sensitivity spectra were noted, and all strains were sensitive to the tetracycline, chloramphenicol, kanamycin, carbomycin, and novobiocin.

In preparation for the treatment of tissue cultures with antibiotics, the minimal inhibitory concentration of several drugs was determined by adding the drug to medium II just before it was poured. The tissue culture fluid from an infected cell line was used as inoculum. Tetracycline was found to inhibit growth completely at 0.5 μg/ml (but not at 0.25 μg), whereas chloramphenicol inhibited PPLO growth at 8 μg/ml (but not at 2 μg). Tetracycline in graded doses was then added to freshly planted bottle cultures of HeLa cells in growth medium (according to the technique described above). At concentrations of 20 μg/ml and 10 μg/ml, tetracycline was clearly inhibitory to growth of HeLa cells. Questionable

TABLE 4

Antibiotic sensitivities

Antibiotic Disc Used	T-5	C-2-7	HeLa (T)	MK 1-58	RK 10-58	Alf.	Uppsala
Penicillin, 10 units	+	+	+	+	+	+	+
Dihydrostreptomycin, 10 μg	+	+	+	+	+	+	+
Erythromycin, 15 μg	+	+	+	+	+	+	+
Bacitracin, 10 units	+	+	+	+	+	+	+
Polymyxin, 30 μg	+	+	+	+	+	+	+
Neomycin, 30 μg	+	+	+	+	+	+	+
Triple sulfa, 1 mg	+	+	+	+	+	+	+
Nystatin, 100 units	+	+	+	+	+	+	+
Vancomycin, 30 μg	+	+	+	+	+	+	+
Oleandomycin, 15 μg	+	+	+	+	+	+	+
Kanamycin, 30 μg	−	−	−	−	−	−	−
Tetracycline, 30 μg	−	−	−	−	−	−	−
Oxytetracycline, 30 μg	−	−	−	−	−	−	−
Chlortetracycline, 30 μg	−	−	−	−	−	−	−
Chloramphenicol, 30 μg	−	−	−	−	−	−	−
Carbomycin, 15 μg	−	−	−	−	−	−	−
Novobiocin, 30 μg	−	−	−	−	−	−	−

+ = Growth of PPLO (no inhibition); − = no growth of PPLO (inhibition).

inhibition of cell growth was noted at 5 μg/ml, but none was noted at 2.5 and 1.25 μg/ml.

A concentration of 2.5 μg tetracycline/ml was then incorporated in the tissue culture medium for four successive passages. It was thought that higher concentrations might alter the cell line by selection of resistant cells. The line has since been carried in the absence of tetracycline for 17 months (75 transfers) and remains free of PPLO. Other cell lines at the Virus and Rickettsia Section have been rendered free of PPLO by a single passage in media containing 2.5 μg tetracycline/ml and have remained free during shorter periods of transfer.

Attempts to learn the origin of PPLO infection of tissue culture. A) It has been proposed on many occasions that PPLO infections of HeLa cells arise as a result of accidental contamination of the tissue culture with ordinary bacteria (B forms) and their conversion to stable L forms indistinguishable from PPLO, as a result of prolonged passage in the presence of penicillin. This proposition was tested by adding B forms of a number of bacterial species to tissue cultures. A HeLa cell line maintained in 10% calf serum, 90% balanced salt solution containing Eagle's amino acids and vitamins, 1 mmole arginine, 200 units penicillin/ml, and 100 μg streptomycin/ml had been rendered free of PPLO by treatment with tetracycline. Tissue culture fluids were changed at 3, 5, 7, 11, and 14 days after inoculation. Three tubes of HeLa cells were inoculated with each culture, one tube receiving a loopful of a heavy suspension, the other two tubes 1:10 and 1:100 dilutions. The cultures tested had been isolated from human throats in the course of a study of respiratory disease and had been transferred only a few times. They were kindly provided by Harold Kaye of the Respiratory Unit of the Virus and Rickettsia Section. The cultures were: an α-hemolytic streptococcus, a β-hemolytic streptococcus of group A and one culture each of group B, C, D, and G, a γ-hemolytic streptococcus, *Neisseria* sp., *Staphylococcus aureus*, *Pseudomonas* sp., a diphtheroid, *Diplococcus pneumoniae* (untyped), and a *Candida* sp. All strains of bacteria were selected to be sensitive to one of the antibiotics. Although bacterial growth destroyed a few of the tissue cultures, most remained in good condition until the end of the experiment. Tissue culture fluids were tested at 3, 7, and 14 days on medium I. PPLO were not cultured in a single instance.

B) It seemed possible that PPLO infections of tissue cultures might arise from salivary contamination in the laboratory, perhaps during pipetting. Saliva specimens were collected from all six persons working with tissue cultures; included were those who had worked with two cell lines and two virus strains originating in this laboratory and subsequently found to be infected with PPLO. Two tubes each of monkey kidney tissue cultures were inoculated with each specimen diluted 1:10, 1:100, and 1:10,000. The tissue culture medium was 10% calf serum plus 90% balanced salt solution containing Eagle's amino acids and vitamins, 1 mmole arginine, 0.1 mmole glycine, 200 units penicillin/ml, and 100 μg streptomycin/ml. It was changed three times weekly. Obvious gross bacterial contamination occurred in some instances, and the tubes were discarded, but 22 of the 36 cultures survived the 19-day period. Each tube was cultured before inoculation and at 19 days. PPLO were not demonstrated in any of the specimens. The only organism grown on the plates was a diphtheroid.

The same specimens were inoculated in 0.05 ml amounts on medium III containing 100 units penicillin/ml and 50 μg streptomycin/ml. No PPLO colonies were observed.

C) In the course of a study of the etiology of acute upper respiratory diseases, a more extensive survey of the presence of PPLO in upper respiratory passages was accomplished. Throat washings (10 ml of 5% nutrient broth) collected from persons with these infections were inoculated in 0.2-ml amounts into monkey kidney tissue cultures and passaged several times usually at about 7-day intervals. Third and fourth passage material (kindly supplied by Roslyn C. Robinson of the Virus and Rickettsia Section) was plated on medium I. The material from 97 patients was tested in this manner and only 1 was found to contain PPLO.

D) It has been suggested that PPLO arise from the serum constituent of the tissue culture medium (Holmgren and Smith, 1959). The maintenance of the HeLa cell line free of PPLO for 17 months in a medium containing 20% human serum and 10% bovine fetal serum has constituted a stringent test of this proposition. The routine employed has resulted in the ex-

posure of each bottle to 10 ml of human serum in each passage or 750 ml in 75 transfers. During the 17 months in question, 4 serum pools have been used, each pool representing about 20 people. Thus there has been no experimental support of this suggestion.

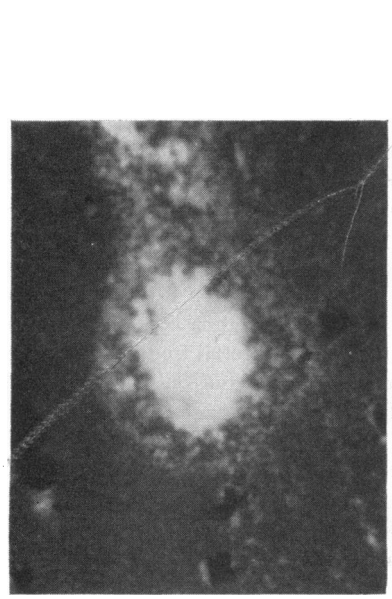
Fluorescent antibody studies. It was of interest to visualize the PPLO in the cell layer and the fluorescent antibody technique was appropriate for this purpose. Strain T-5 was grown on medium I containing rabbit blood in place of human bank blood. After 5 days the colonies were washed and scraped into 2 ml of balanced salt solution. Colony counts indicated that at least 7×10^7 PPLO were harvested from a plate. The same rabbit that was bled to prepare the plates was immunized by four intramuscular injections 3 to 5 days apart, each injection representing the washing from one plate. The animal was bled 12 days after the final injection, and the globulins labeled with fluorescein isothiocyanate. The fluorescent antibody procedures employed have been described (Goldwasser and Shepard, 1958; Carski, 1960). Adsorption of the conjugate was with rabbit liver, hamster kidney, and rabbit kidney tissue powders.

Colonial growth from plates was examined by placing cover slips on the plates, and then gently raising the cover slip with forceps. After air-drying for 15 min, the cover slips were stored at −65 C, and before staining they were fixed in acetone at room temperature for 10 min.

Primary monkey kidney and rabbit kidney cover slip cultures were inoculated with a loop with T-5 colonies from a plate, and at intervals cover slips were washed once with balanced salt solution, air-dried for 30 min, then treated in the same manner as smears of colonial growth from plates described above.

Proofs of specificity of staining were the following: (i) A heterologous conjugate from a rabbit immunized with human globulin did not stain the colonies; (ii) exposure of the smears to unlabeled anti-T-5 globulin for 30 min, followed by a staining period of 10 min, produced inhibition of staining, whereas exposure to normal rabbit globulin under the same conditions did not inhibit staining; (iii) diluting the anti-T-5 conjugate in a suspension of T-5 organisms washed from a plate with balanced salt solution caused inhibition of staining in comparison to the same dilutions in washings from uninoculated control plates of the same culture medium.

The impression smears from agar plates, when stained with homologous fluorescent antibody, contained brightly stained colonies. The morphology resembled that revealed by Giemsa stains. Round, vacuolar structures that did not



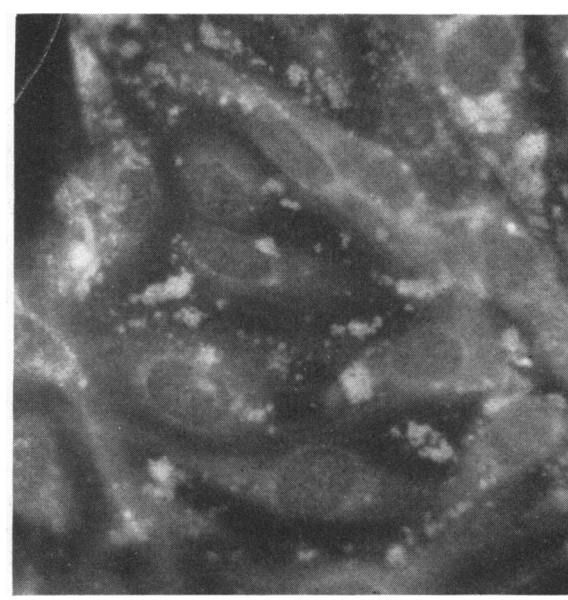


Fig. 2 (left). PPLO colony impression smear stained by fluorescent antibody (×365).
Fig. 3 (right). PPLO antigen in monkey kidney tissue culture (×365).

stain intensely were usually present. Small, discrete, brightly staining bodies could be readily seen, especially at the edge of intact colonies or in the remnants of disrupted colonies. They were too small to define morphologically, and were not readily visible in heavily stained portions (Fig. 2).

Tissue culture monolayers inoculated with PPLO contained clumps of particles scattered throughout (Fig. 3). They were randomly located with regard to cell structure, and showed no tendency to perinuclear location as do intracellularly grown bacteria (Shepard, 1957, 1959). Some clumps of antigen were located on the bare coverslip between the cells. Although much of the PPLO antigen was located on the cell surface or extracellularly, it was not possible to rule out the possibility that part of it was intracellular.

These results with fluorescent antibody are compatible with the electron microscope studies of Edwards and Fogh (1960) who in sectioned material found most of the PPLO to be located on the cell membrane or in intercellular spaces. They observed the size of the PPLO to be 310 by 450 mμ, which is too small to allow one to distinguish shape reliably by light microscope methods.

DISCUSSION

Tissue culture media devised for optimal growth of human cell lines did not by themselves allow multiplication of PPLO. When the HeLa cells were present, however, the PPLO grew to a high concentration. It seems possible that this nutritional dependency could arise from a situation in which the HeLa cells provide the PPLO with nutrilites that are absent from the tissue culture medium, or one where the animal cells inactivate substances that prevent growth of the PPLO. The nutritional relationship appears to be an intimate one since the chief site of growth of PPLO is very probably the outer surface of the animal cell membrane. This particular location is evidenced by the electron micrographs of Edwards and Fogh. It is also attested by the fluorescent antibody results, which, in providing a more general picture, show clumps of PPLO scattered randomly across the surface of the cell. This distribution is quite different from that seen in these cells with intracellularly grown bacteria, which are characteristically concentrated in the perinuclear region, especially in cells that are isolated enough to be spread out. The occasional PPLO seen by Edwards and Fogh to be surrounded by cytoplasm is not evidence by itself that the organisms grew there.

The interesting question as to the origin of the PPLO infections of cell lines remains unanswered. It has been reported that PPLO were isolated from 30 to 40% of human throats and from 46% of human saliva specimens (reviewed by Morton (1958)). Our findings were that PPLO capable of growing in tissue culture are found very infrequently in human throats. Ninety-seven human throat washings were passed in tissue culture as part of a study of human respiratory disease and only one isolation of PPLO resulted. More specifically, the people working with tissue cultures in this laboratory were not found to have PPLO in their saliva. No experimental support was obtained for the suggestions that PPLO infections arise from the serum constituent of the medium, or that bacterial contaminants (B forms) are converted to PPLO by action of the antibiotics. The latter suggestion has recently been repeated (Holmgren and Campbell, 1960), but the experimental support referred not to a PPLO but an L form of an accompanying gram-negative rod that was also growing in the tissue culture.

The practices commonly used to maintain cell lines are, of course, well suited for the maintenance of their PPLO infections also. The very great number of manipulations carried out over the years of their continuous existence in vitro have exposed them to microbial contamination from many sources. The efficacy of the antibiotics, penicillin and streptomycin, in controlling many of the common bacterial contaminants, has probably led in many laboratories to a relaxation from bacteriologically sterile techniques, which are based on the principle that contaminants are to be prevented from entering the experimental system. Perhaps now that cell lines may be preserved for long periods by freezing to very low temperatures, it would be better to omit antibiotics from the media used to grow the basic cell line stock, so that the appearance of the usual bacterial contaminants could be used as a signal of breaks in technique.

In routine operations where convenience is the most important consideration, antibiotics must

be employed. Under these conditions, the control of PPLO infections of cell lines is not difficult and there seems to be no good reason to allow this source of confusion to cloud future results. Our current practice is to culture an aliquot of the cell suspension at each transfer of the cell line. The PPLO medium is simple to prepare, and growth, if present, is easily detected. Although it has been reported that high and clearly cytotoxic concentrations of tetracyclines are necessary to eliminate PPLO (e.g., 100 to 200 μg/ml (Hearn et al., 1959)), in our experience, 2.5 μg tetracycline/ml, when present in the early growth phase, were completely effective. It seems possible that the high reported requirement was the result of attempting to treat mature cell monolayers where the PPLO would be present in high concentrations at a period after their active growth phase. Concentrations below the cytotoxic level seem preferable to avoid changes in the properties of the cell line.

SUMMARY

The medium of Barile, Yaguchi, and Eveland was found to be a satisfactory medium for the culture of pleuropneumonia-like organisms (PPLO) in cell lines. Somewhat simpler to prepare and at least as efficient was 15% human blood added to blood agar base. A transparent medium giving even better growth contained human serum, yeast, and red blood cell extracts. As a liquid medium it supported growth of PPLO with a generation time of 1.3 hr. Tissue culture medium in the absence of cells did not support the growth of PPLO.

Most of the cell lines at this laboratory were infected with PPLO. Most of the virus strains propagated on cell lines and some of those grown in primary tissue cultures contained PPLO.

Growth curves of PPLO in tissue culture were studied. In 48 hr the number of PPLO rose to a concentration of 10^7/ml in the fluid, and probably even more of these organisms were present in association with the cells.

Fluorescent antibody stains showed much PPLO antigen in clumps in a distribution that accorded with a location on the cell membrane.

The antibiotic spectrum of a number of isolates of PPLO was studied with antibiotic sensitivity discs and no differences were found among them. The minimal inhibitory concentration for PPLO and the minimal toxic level for HeLa cells for tetracycline were determined. To rid a cell line of its infection, 2.5 μg tetracycline/ml were incorporated in the medium for one passage. One cell line has now remained free of PPLO for 17 months (75 transfers) without further treatment with tetracycline.

It was not possible to institute PPLO infections of cell lines by inoculating them with bacteria of a number of species, all susceptible to penicillin or streptomycin. The addition of saliva of laboratory personnel to the tissue cultures was likewise ineffective in starting PPLO infections. Serum seems not to be a frequent source, since the PPLO-free cell line has now been exposed to 750 ml of human serum from four different lots, each lot representing 20 or more people.

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