Detection and Possible Source of Contaminating Pleuropneumonialike Organisms (PPLO) in Cultures of Tissue Cells. (24533)

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One line of conjunctival cells and 3 of 5 lines of HeLa cells were reported by Robinson *et al.*(1) to be contaminated with pleuropneumonialike organisms (PPLO) even though the cultures routinely contained 100 units of penicillin and 100 μg of streptomycin /ml of medium. Collier(2) found 11 of 14 lines of HeLa cells contaminated with PPLO. The 3 lines which were not contaminated had at some time been cultivated in the presence of aureomycin, terramycin or neomycin. This report deals with research directed towards determining the extent of PPLO contamination of tissue cell cultures and source of this contamination.

Materials and methods. Samples of tissue cell cultures were obtained from 5 laboratories as listed in Table I. Multiple samples containing both cells and supernatant were taken at varying periods during growth of the tissue cells. The tissue cells were grown in Hank's, Eagle's or medium 199 containing 10 to 20% horse, human, calf, rabbit or dog serum. One ml of tissue cell culture containing both cells and supernatant was transferred to 5 ml of Bacto-PPLO broth(3) enriched with 1% PPLO serum fraction(4). After 3 days aerobic incubation at 37°C, 0.2 to 0.4 ml of this culture were inoculated over the entire surface of Bacto-PPLO agar(3) enriched with 1% PPLO serum fraction. Plate cultures were incubated aerobically at 37°C. In some cases duplicate tests were made by inoculating tissue cell cultures directly onto the agar medium. From the third to seventh day the inverted Petri dish cultures were examined by transmitted light under low power of microscope (100 ×). Presence of PPLO colonies was established by attempting to move the colonies with a wire loop, staining with Dienes stain, and repeated sub-culture on agar medium. Samples of both fresh and frozen horse, human, and calf serum were obtained from stock supplies used at the various laboratories. Both individual and pooled samples were tested for PPLO by inoculating 1 ml of sample into 5 ml of PPLO broth enriched with 1% PPLO serum fraction. These cultures were incubated 3 days at 37°C then 1 ml was transferred to a second tube of same medium. After 3 more days incubation the broth cultures were plated, incubated, and examined as previously described for testing of tissue cell cultures.

Results. Colonies of PPLO appeared on the surface of agar medium 3 to 5 days after inoculation. The colonies, upon primary isolation, varied considerably in size and morphology. The diameters of colonies ranged from 0.08 mm to nearly 0.3 mm. The morphology varied from vacuolated, lacy, to small dense refractile types. After repeated passage on agar medium the colonies assumed the classical fried egg appearance (Fig. 1). Colonies stained with Dienes stain and would not rub off the surface of the agar. All PPLO contaminants which were isolated required serum or serum fraction for growth.

Direct seeding of tissue cell cultures onto the agar medium did not increase the frequency of positive isolations. PPLO colonies, which were not as numerous as after one passage in broth, were more difficult to recognize among the debris of tissue cells.

Frequently strains of PPLO did not grow well until after 3 or 4 serial transfers on agar medium. Occasionally, when a tissue cell line had been cultured repeatedly, a negative result was obtained among a series of positive isolations. This probably reflects unavoidable faults in sampling or cultivation.

Of 37 cell lines tested, 22 were contaminated with PPLO. Cell lines derived from human, monkey, and mouse tissues were positive. All cells in first or second passage were negative. PPLO were isolated from cells growing in human, horse, and calf sera. All but 3 positive cell lines had at some time been grown in presence of human serum. Two lines of monkey kidney cells and a line

TABLE I. Cell Cultures Examined for Presence of Pleuropneumonialike Organisms.

Cells	Type	Source*	Serum	Passage	Lines + / lines tested
HeLa	human carcinoma	1	human	40– 64	1/1
"	" "	2	" & calf	25–121	7/10
"	" "	3	" horse		1/1
H. Conj.	" conjunctiva	1	" "	88–100	"
H. K.	" kidney	1	" "	99–111	"
C_3	" fibroma	1	"	11	"
Int.	" embryonic intestine	1	"	40– 48	"
KB	oral cancer	2	"	22	"
Lung	human lung	3	" & horse		"
MCN	" bone marrow	3	" "		"
Hep_2	" epithelioma	1	"	10	"
Hep_1	" "	2	"	15	0/1
Am	" amnion	2	" & calf	20	1/2
Chang L	" liver	2	"	11	1/1
MK	monkey kidney	4	horse	100–200	2/2
"	" "	1	calf	1	0/1
"	" "	3	human	1	"
SCH	" heart	1	calf	70	"
L	mouse mesenchymal	4	horse	100	1/1
ERK	embryonic rabbit kidney	1	rabbit		0/1
RBK	adult " "	1	calf		"
RK	*Idem*	1	human & calf	1– 2	"
"	"	3	calf	1	"
BK	bovine kidney	1	"		"
PK	porcine "	1	"		"
Dog	dog fascia	5	dog	22	"

* 1 = So. Jersey Med. Research Foundation, Camden, N. J.
2 = Univ. of Miami School of Med.
3 = Children's Hosp., Philadelphia.
4 = Connaught Medical Research Labs., Univ. of Toronto.
5 = Presbyterian Hosp., Philadelphia.

of mouse mesenchymal cells, which were never in contact with human serum, were contaminated with PPLO.

None of the contaminated cell lines demonstrated gross cytopathogenic effects and no turbidity was evident. In some lines it was noted that the infected cells failed to adhere to the glass surface in continuous sheets.

All 25 samples of sera tested were negative for PPLO. These included 7 pooled lots of horse serum, 2 pooled samples of calf serum and 16 samples of human serum comprising pooled lots and individual samples.

Discussion. Extensive contamination of tissue cell cultures by PPLO could have profound effects on research in which tissue cells are being studied or used as tools. Presence of PPLO in tissue cell cultures has been shown by Coriell *et al.* (5) to account for some serological cross reactions between different lines of tissue cells. The effects of PPLO contamination on cell metabolism and viral growth in cells remains to be studied.

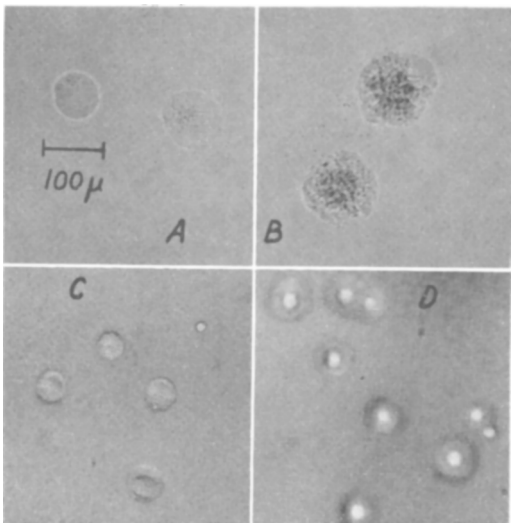

FIG. 1. A. Vacuolated type of colony of PPLO on first passage on solid medium from oral cancer cell line. B. Lacy type of colony in same culture as A. C. Dense type of colony from strain of PPLO isolated from HeLa cells. D. Fried egg appearance of PPLO colonies in same culture as C. All cultures were 8 days old when photographed. 80×.

Failure of tissue culture PPLO to cause cytopathogenic effects or turbidity makes culturing a necessity. Shepard(6) and Wittler *et al.*(7) reported intracellular growth of human genital strains of PPLO and Nelson(8) demonstrated intracellular growth of mouse strains of PPLO. Preliminary observations indicate that this is also true in the case of PPLO contaminants of tissue cell cultures. This feature should be kept in mind when attempting to free tissue cell cultures of their contaminating PPLO.

The source of the contaminating PPLO is unknown. It was suggested by Robinson *et al.*(1) that presence of PPLO in tissue cell cultures was probably due to contamination of some common component of culture media. Although most contaminated cell lines have been in contact with human serum, 3 positive isolations have been made from cells grown in horse serum and which had never been in contact with human serum. These isolations together with failure to isolate PPLO from samples of human serum as reported here, and previous failures to isolate PPLO from routine blood cultures(9,10) indicates that serum is probably not the source of the contaminants.

Collier(2) suggested that the cell lines were contaminated with PPLO when originally isolated. This hypothesis could explain contamination of some cell lines, for PPLO are frequently isolated from human genitourinary tract(9) and oral cavity(11,12). This suggestion, however, would not explain the presence of PPLO in strains of cells derived from such sources as human bone marrow and liver, monkey kidney and mouse mesenchymal tissues, or for greater frequency of contamination in cell lines which have undergone numerous passages.

Direct introduction into tissue cell cultures of human PPLO by a break in aseptic technic could account for the PPLO contamination. Serological comparisons now under way may help to clarify the role of human strains of PPLO in contamination of tissue cell cultures.

It is unlikely that the PPLO were introduced into tissue cell lines during testing, for the contaminants have not serologically reacted, as far as tested, with our stock laboratory strains of PPLO.

Another possible source of contamination is by formation of stabilized L forms of contaminating bacteria by action of penicillin which is widely used in tissue cell cultures. Formation of stable L forms has been demonstrated with *Streptococci*(13), diphtheroids (14), *Streptobacillus moniliformis*(15), *Proteus*(16), and *Salmonella*(17). Reversion of a human genital PPLO to a *Corynebacterium* has been reported(7) during tissue culture passage and conversion of a stock strain of human genital PPLO to a diphtheroid has been demonstrated(18). Minck(19) reported that 5 strains of PPLO isolated from female genital tract developed into diphtheroids and McKay and Taylor(20) described reversion of poultry strains of PPLO to bacteria similar to *Hemophilus gallinarum*. By definition, the difference between L forms of bacteria and PPLO is that L forms are known to be associated with some bacterial species while PPLO are not. Bacteria are common contaminants in tissue cell cultures and it is possible that the PPLO contaminants represent stable L forms produced from the contaminating bacteria by the action of penicillin.

Summary. 1) A total of 37 tissue cell lines growing in the presence of horse, calf, human, rabbit or dog serum have been tested for contamination by PPLO. Of these, 22 or 59% contained PPLO. Human, monkey, and mouse cell lines were contaminated. All first and second passages of cell lines were negative. PPLO were isolated from tissue cells growing in human, horse or calf serum. Nineteen positive cell lines were, at some time, grown in presence of human serum. Three positive lines growing in horse serum had never been in contact with human serum. 2) All 25 samples of sera, including pooled lots of human, calf, and horse sera, were negative for PPLO. 3) Possible sources of PPLO contamination of tissue cell cultures are discussed.

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Effect of Insulin on Glucose Uptake by Mouse Diaphragm Tissue. (24534)

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The effect of insulin in increasing glucose uptake by isolated rat diaphragm tissue has been employed by various workers(1-7) for estimating minute amounts of insulin. Our preliminary results in determining insulin activity using the rat hemidiaphragm technic were so often variable that we undertook the present study to develop a more reliable test method. We believe the method to be described employing pooled mouse hemidiaphragms is an improvement over the rat diaphragm technic from the standpoint of sensitivity and reproducibility. In developing the method, a systematic study was made of the various factors affecting glucose uptake to assess their effect upon the assay of insulin.

Materials and methods. Swiss-Webster and NIH general purpose male albino mice were used in most of our studies. A few experiments were made with DBA-2 and C-57 strains of mice. No difference in insulin responsiveness which could be attributable to strain differences was found. For each experiment the mice were of same weight ($\pm$ 2 g) and in most experiments were in the 25-30 g weight range. Mice were fasted for 16-24 hours before use. Unless otherwise stated, the medium employed for incubation was a modified Krebs-Ringer-bicarbonate solution, hereafter referred to as KRB solution, having the following composition in millimoles/l: NaCl (95), KCl (4.7), $CaCl_2$ (2.5), KH_2PO_4 (1.2), and $NaHCO_3$ (50). Glucose was added to give a concentration of 1 mg/ml. The insulin used was a glucagon-free, crystalline zinc sample (Eli Lilly & Co.) which assayed 24.6 units/mg. Dilutions of a stock insulin solution (10 units/ml) were made before each experiment with 0.9% saline solution which had been brought to pH 3 with 1 N HCl. A systematic procedure was followed in preparation of the diaphragms. Groups of 5 mice were treated successively in the following manner. As each mouse was killed by decapitation its diaphragm was excised with a pair of iris scissors and placed in ice cold KRB solution (without glucose) which was gassed continuously with a mixture of 95% O_2-5% CO_2.