ens of both sexes approximated a biological half life of 29.9 days. In dogs of both sexes, plasma disappearance rate was very rapid (T ½ = 1.5 days). Tissue radioactivity varied between tissues of the same species and between the various species studied.

1. Unglaub, W. G., Rosenthal, H. L., Goldsmith, G. A., *J. Lab. Clin. Med.*, 1954, v43, 143.

2. Rosenthal, H. L., Hampton, J. K., Jr., *J. Nutrition*, 1955, v56, 67.

3. Mollin, D. L., Ross, G. I. M., *J. Clin. Path.*, 1953, v6, 54.

4. Miller, A., Corbus, H. F., Sullivan, J. F., *J. Clin. Invest.*, 1956, v36, 18.

5. Rosenthal, H. L., *J. Nutrition*, 1959, v68, 297.

6. Rosenthal, H. L., Brown, C. L., Jr., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 117.

7. Gregory, M. C., Holdsworth, E. S., *Biochem. J.*, 1959, v72, 549.

8. Ross, G. I. M., *J. Clin. Path.*, 1952, v5, 250.

9. Reizenstein, P. G., *Acta Med. Scand.*, 1959, v165, 1.

10. Rosenthal, H. L., *Am. J. Physiol.*, 1959, v197, 1048.

Received June 3, 1960. P.S.E.B.M., 1960, v105.

Isolation and Elimination of Pleuropneumonia-Like Organisms From Mammalian Cell Cultures.* (25992)

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Following the report of Robinson, *et al.*(1) on association of pleuropneumonia-like organisms (PPLO) with human cells in culture, high incidence of PPLO contamination has been found in several laboratories(2-4). Our findings have been briefly presented in preliminary communications(5,6). Methods for surveillance of animal cell cultures for PPLO and for their elimination are important to application of cell cultures in virological and physiological research, since PPLO contamination is not revealed by grossly visible changes in culture appearance or cellular morphology. Neither are influences of PPLO on metabolism of animal cells in culture known. This paper reports results of a program instituted in the Dept. of Bacteriology, Univ. of Minnesota, for isolation and cultivation of PPLO from established animal cell lines. Use of the antibiotic kanamycin for elimination of PPLO from contaminated cell lines is described.

Materials and methods. Cells tested were mostly those propagated in this laboratory in continuous culture for varying periods from 2 to 5 years. Twenty-four lines submitted by other investigators were also tested. Since results were communicated individually to submitting investigators, these results are combined with other data for presentation here. Fifteen lines, all but one propagated by the same worker for more than 2 years, were used for evaluation of the effectiveness of kanamycin. Cell lines from Minnesota were propagated as described(7), while other cells were cultivated in routinely employed media. All media contained human, horse, calf or rabbit serum supplemented with a) yeast extract in Hanks' balanced salt solution or b) Eagle basal medium(8). Serum pools were Selas-filtered and inactivated at 56°C for 30 minutes before use. *Trypsin* (Difco 1:250) in 10 times stock solution was prepared by dissolving 0.2 g in 100 ml of GKN solution (5.5 mM glucose, 5.4 mM potassium chloride, 138 mM sodium chloride). Trypsin stock was adjusted to pH 7.8 with sodium bicarbonate (166 mM) solution, sterilized by Selas filtration, and stored at –20°C. *Kanamycin sulfate*† was dissolved in Hanks' balanced salt solution to a concentration of 10^4 μg kanamycin base per ml.

PPLO medium, modified from the formulae

* Aided by grants from Am. Cancer Soc., Nat. Foundation and Nat. Cancer Inst., Nat. Inst. Health, P.H.S.

† Kanamycin sulfate was supplied kindly by Dr. C. H. Mann, Bristol Labs., Inc.

of Brown(9) and of Robinson and Wichelhausen(10), was prepared by mixing 250 g ground beef heart with 900 ml of distilled water and 5 g sodium chloride. Pancreatin (Baltimore Biol. Labs., lactose-free), 2.5 g, was dissolved in 100 ml of 0.5% sodium chloride and added to the beef-water mixture after it was pre-warmed in a water bath to 50-56°C. After digestion for 2 hours with frequent agitation the mixture was filtered through 4 layers of gauze, and the filtrate boiled for 5 minutes and filtered through coarse filter paper. The solution was heated to boiling, restored to volume and filtered through Whatman No. 2 paper. The broth was enriched with 1 g yeast extract (Difco) per liter, cooled, adjusted to pH 8 with 1 N sodium hydroxide solution, and dispensed in amounts suitable for autoclaving at 121°C for 15 minutes. For solid medium, 1.5% Difco special agar (Noble) was added to the broth and dissolved by boiling. After autoclaving and before use, both liquid and solid media were enriched with 20% filtered human ascitic fluid previously tested for capacity to support growth of PPLO.

Results. Isolation of PPLO from cell lines in continuous culture. Cultures of 166 sublines of 68 cell lines were tested for PPLO contamination over a period of 18 months. Cultures were propagated in 1 ml of medium in screw-cap tubes, and were fed twice weekly by complete medium replacement. Cell suspensions prepared for passage of cell cultures, fed 24 hours previously, were employed for isolation of PPLO. Monolayers were rinsed with GKN and dispersed in 1 ml of 0.02% trypsin allowed to act for 1-2 minutes. After dispersion by pipetting, cells were sedimented at 40-50 x gravity for 3-5 minutes. The supernate was discarded and the cells were suspended in conditioned medium containing sufficient fresh growth medium for establishment of new cultures. From 1 ml of this suspension, 0.1 ml was immediately inoculated into 5 ml of Difco trypticase soy broth containing 0.1% agar, 0.02 ml streaked on an agar plate of the PPLO test medium, and the remainder inoculated into PPLO broth medium. After 24 hours at 37°C PPLO broth and agar subcultures were prepared from the inoculated tube of PPLO broth. After 24 hours more incubation, the first and second PPLO broth cultures were inoculated on agar medium. Plates were examined with a stereoscopic microscope daily for the first 4 days and every day thereafter for a period of 2 weeks. Trypticase soy broth cultures were examined routinely for turbidity as an indication of ordinary bacterial contamination. Data are given in Table I. Results of repeated tests of given cell sublines were consistently negative or positive for presence of PPLO, with the exception of variable results observed with 5 sublines. PPLO isolated from 2 of the sublines listed in Table I, clonal HeLa 4 and altered monkey kidney(11), grew sparsely and slowly. With most but not all cell lines, at least one subline was found uncontaminated to serve for regeneration of stock; for decontamination of the remainder, an antibacterial agent effective against PPLO but nontoxic for cells was desirable.

Elimination of PPLO contamination with kanamycin. Preliminary survey of antibiotics and chemotherapeutic agents showed that most of the broad-spectrum antibiotics were effective against PPLO in high concentration. Only kanamycin(12) could be used in relatively high concentration without detrimental effect on cell morphology and growth. Fifteen PPLO-contaminated cell sublines were used to study the value of kanamycin for decontamination. Six replicate cultures per line were established by incubation for 3 days. Medium was replaced completely with fresh growth medium; 3 of the tube cultures received 100 μg of kanamycin in the 1 ml of replacement growth medium. Twice during the following treatment period, 1 control and 1 treated culture from each line were redispersed with trypsin and used for propagation of 3 new cultures each. Three days after the second such passage, kanamycin treatment was discontinued and both control and treated lines maintained independently thereafter in identical media. Total period of treatment was 3 weeks. Cells were tested for PPLO contamination 12 days after discontinuation of treatment, and retested at approximately monthly intervals thereafter. Results listed in Table II show that in no case was a treated

TABLE I. Summary of Results of Cultures for PPLO from 166 Sublines of Mammalian Cells.

Source	Total sublines studied	Negative		Positive		Variable		
		No. sublines	Total cultures	No. sublines	Total cultures	Subline	Cultures Pos	Neg
Human	110	45	207	63	258	Minn. HeLa E	13	2
						Clonal Minn. HeLa 4	8	6
Rabbit	39	12	32	25	31	Minn. 58-3-1 (E)	2	1
						Minn. 58-5-3 (E)	1	1
Monkey	5	3	15	1	2	Altered monkey kidney	7	4
Mouse	2	2	6					
Porcine	5	5	17					
Bovine	2	2	12					
Misc.	3	3	14					
Total	166	72	303	89	291	5	31	14

culture found positive. Control cultures continued to yield PPLO consistently with one exception, the clonal Minnesota HeLa, which carries a strain of PPLO that grows slowly and is difficult to isolate. The PPLO-free cell sublines obtained by this kanamycin treatment have remained free of PPLO for over 10 months. Kanamycin has since been employed successfully for elimination of PPLO from other cell lines. Since the studied lines represent a heterogeneous group obtained from various sources and cultivated in different media, the findings suggest that kanamycin treatment may be broadly useful for elimination of PPLO from contaminated animal cell cultures. PPLO isolated from untreated contaminated cell lines are shown in Fig. 1; a spectrum of morphological colonial types is represented.

Intensity of kanamycin treatment for PPLO elimination. A series of tube cultures, initiated with 25,000 cells per ml culture medium, of a clonal subline of the Minnesota cell line 55-12-1 of human esophageal epithelial

TABLE II. Results of PPLO Cultures from 15 Kanamycin-Treated* Mammalian Cell Lines and Parallel Controls.

Cell line	Medium†	Ref	Control‡		Treated‡	
			Pos	Neg	Pos	Neg
Horse HeLa 100	A	13	10	0	0	10
Idem	B		10	0	0	10
MBA HeLa	B	14	10	0	0	10
HeLa E	C		12	0	0	10
" KC	C		9	0	0	9
Clonal HeLa 4	C		7	4	0	9
" EE (Minn.)	C		10	0	0	10
Chang conjunctiva	A	15	10	0	0	10
Clonal Chang conj. B_2a_2	C		4	0	0	4§
" " " B_2a_4	C		10	0	0	10
Lass liver (Minn.)	C	16	10	0	0	10
Clonal Chang liver A_1a_1	C	15	10	0	0	10
" " " D_1b_1	C		9	0	0	9
" " " A_2b	C		9	0	0	10
U-12	A	17	9	0	0	9

* Treatment begun 6/26/59 and discontinued 7/17/59.

† Medium: A = HoS–20, YEM–80; B = HoS–40, BSS–60; C = HuS–20, YEM–80.
HoS = horse serum, HuS = human serum, BSS = Hanks' solution, YEM = 0.1% yeast extract in BSS with 2.5 mM $NaHCO_3$ when used with HuS or 4.2 mM $NaHCO_3$ when used with HoS.

‡ Cultures performed monthly July 1959 to June 1960.

§ Kanamycin-treated culture lost through contamination after 10/7/59.

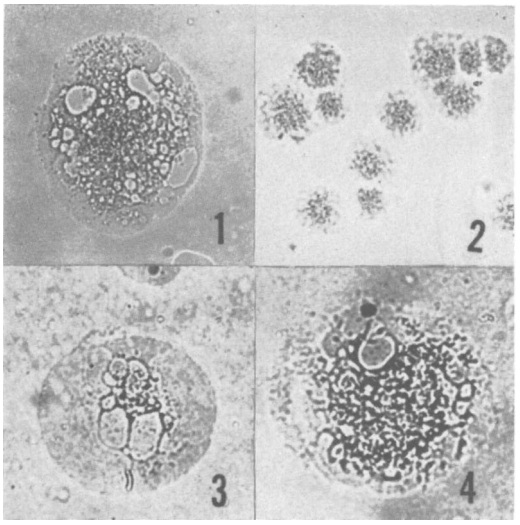

FIG. 1. Colonies of PPLO isolated from mammalian cell cultures. 1. Minnesota HeLa. 2. Clonal Chang liver A_1a_4. 3. Horse HeLa 100. 4. Clonal Chang conjunctiva B_2a_4. × 210.

cells (EE) was treated with kanamycin in graded concentration. At intervals, cultures were rinsed 3 times with balanced salt solution to remove antibiotic; cells were grown for 1 week in antibiotic-free medium, and cultures were trypsin-dispersed for detection of PPLO. Results are tabulated in Table III. It is evident that the time of kanamycin treatment for elimination of PPLO from this cell line could be shortened considerably depending on antibiotic concentration. In contrast to the 3-week treatment arbitrarily used in the first study, 48 hours exposure to 100 μg per ml of kanamycin appeared to present an adequate treatment period with a 4-fold safety margin. Repetition of this experiment gave similar results, as did a similar experiment with the Minn. 56-6-2 line (Lass) of human liver cells(16).

Discussion. Our experience with the described medium and technic for isolation of pleuropneumonia-like organisms from animal cell cultures suggests consistent sensitivity. Although we were concerned mainly with detection of PPLO contamination in our own cell lines and repropagation of uncontaminated lines rather than comparative evaluation of various media and technics, it may be helpful to report that an agar medium commonly employed for isolation of PPLO was not found sufficiently adequate, and that freshly trypsinized cells from monolayer cultures suspended in their growth medium were superior material for consistent isolation of PPLO. Our findings that 65 sublines of human cells in continuous culture were contaminated, as well as 29 sublines of other species, reinforces the experience of other laboratories that PPLO contamination is a widespread danger in cell culture studies. Circumstantial as well as serological(18) evidence indicates that PPLO strains of human origin are a major source of this contamination. Although a high proportion of our cell lines were contaminated with PPLO, evidently a number of lines had been maintained uncontaminated for years. Variation in incidence of PPLO contamination of cell lines maintained in different workrooms of our laboratory was suggestive but did not provide definitive evidence for particular routes of entry of con-

TABLE III. Recovery of PPLO from Contaminated Minn. EE Cell Cultures Treated Variably with Kanamycin.

Medium conc. of kanamycin, μg/ml	Hr of treatment*										
	.5	1	2	3	4	6	10	24	36	48	102
1,000.0	+†	—	—	—	—	—	—	—	—	—	—
320.0	+	+	—	nd	+	—	—	—	—	—	—
100.0	+	+	+	+	—	+	—	—	—	—	—
32.0	+	+	+	+	+	+	—	—	—	—	—
10.0	+	+	+	+	+	+	+	V	V	V	V
3.2	+	+	+	+	+	+	+	+	+	+	+
1.0	+	+	+	+	+	+	+	+	+	+	+

* Replicate monolayer tube cultures exposed to kanamycin in culture medium; after exposure, cultures washed 3 times with BSS, grown 1 wk on growth medium without antibiotic, and dispensed for passage and culture for PPLO.

† + = isolation of PPLO at first and second culture passage after kanamycin treatment; — = failure to isolate PPLO in 2 attempts; V = positive results for one attempt and negative results for others; nd = not done.

taminants into cultures. It was encouraging to find that adequate surveillance was possible by use of the pancreatic digest medium for PPLO isolation, and that decontamination could be accomplished with an antibiotic well tolerated by animal cells. If the efficacy of brief kanamycin treatment is confirmed by continued study of treated cell lines, decontamination with kanamycin will be additionally attractive. Such brief treatment is desirable to permit evaluation of any selective effects of kanamycin on culture evolution, not reflected in visible morphologic influence. Treatment with relatively high levels of kanamycin also is desirable to prevent emergence of antibiotic-resistant PPLO strains. The data of Smith *et al.*(19) indicate that at least 1 human cell line, the Eagle strain KB, is not inhibited in growth by kanamycin in concentration less than 500 μg per ml; the 100 μg per ml level employed in our studies therefore may be well within safe limits for cells. It should be emphasized that the cultures studied here were propagated routinely in medium not containing penicillin or streptomycin. If isolated PPLO are in reality L forms induced by prior use of these antibiotics during culture propagation, then maintenance of kanamycin-treated cell lines without recontamination is understandable. If, alternatively, the PPLO are organisms of human origin as suggested by serological evidence from other laboratories, then successful maintenance following kanamycin treatment of cultures indicates that PPLO contamination can be avoided by rigorous aseptic cell-cultural technic. Prolonged propagation of treated cultures without antibiotic suggests that properly prepared serum is not a likely source of contamination. Although we have not yet encountered kanamycin-resistant PPLO in cell cultures, the possibility suggests that kanamycin should not be regarded as a prophylactic substitute for adequate preventive technic, but only as a promising therapeutic agent for rescue of valuable contaminated cell lines.

Summary. One hundred sixty-six sublines representative of 68 culture lines of cells of human, rabbit, monkey, mouse, pig, cow and other species origin were tested repeatedly for contamination with pleuropneumonia-like organisms over a period of 18 months. Cells freshly dispersed with trypsin were inoculated into a medium containing pancreatic digest of beef heart enriched with yeast extract and human ascitic fluid. Of the tested sublines, 57% were contaminated with PPLO. Repeated tests with the heart digest medium on 89 sublines were consistently positive, while 5 sublines yielded variable results. PPLO strains isolated from 2 of the variable cell sublines grew sparsely and slowly, but growth of other PPLO strains was luxuriant in the test medium. Fifteen cell lines carrying PPLO of varying colonial morphology were treated for 3 weeks with 100 μg of kanamycin per ml of medium. Treated sublines yielded no PPLO during subsequent cultivation for nearly a year in the absence of kanamycin. A contaminated strain of human esophageal epithelium has been freed of PPLO by brief exposure to kanamycin. Required time of exposure appeared inversely related to kanamycin concentration employed.

1. Robinson, L., Wichelhausen, R. H., Roizman, B., *Science,* 1956, v124, 1147.

2. Collier, L. H., *Nature,* 1957, v180, 757.

3. Rothblat, G. H., Morton, H. E., PROC. SOC. EXP. BIOL. AND MED., 1959, v100, 87.

4. Rothblat, G. H., *Ann. N. Y. Acad. Sci.,* 1960, v79, 430.

5. Pollock, M. E., Kenny, G. E., Syverton, J. T., *Excerpta Med.,* in press.

6. Kenny, G. E., Pollock, M. E., Syverton, J. T., *Bact. Proc.,* 1960, 121.

7. Syverton, J. T., Scherer, W. F., *Ann. N. Y. Acad. Sci.,* 1954, v58, 1056.

8. Eagle, H., *J. Biol. Chem.,* 1957, v226, 119.

9. Brown, J. H., *J. Bact.,* 1948, v55, 871.

10. Robinson, L. B., Wichelhausen, R. H., *J. Lab. and Clin. Med.,* 1952, v39, 290.

11. Rothfels, K. H., Axelrad, A. A., Siminovitch, L., McCulloch, E. A., Parker, R. C., *Proc. 3rd Canadian Cancer Conf.,* 1959, 189.

12. Umezawa, H., Ueda, M., Maeda, K., Yagishita, K., Kondo, S., Okami, Y., Utahara, R., Osato, Y., Nitta, K., Takeuchi, T., *J. Antibiotics Japan Ser. A.,* 1957, v10, 181.

13. Randall, C. C., *Fed. Proc.,* 1955, v14, 416.

14. Scherer, W. F., *Ann. N. Y. Acad. Sci.,* 1955, v61, 806.

15. Chang, R. S., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 440.
16. Syverton, J. T., McLaren, L. C., *Cancer Research,* 1957, v17, 923.
17. Swim, H. E., Parker, R. F., *Am. J. Hyg.,* 1957, v66, 235.
18. Coriell, L. L., Fabrizio, D. P., Wilson, S. R., *Ann. N. Y. Acad. Sci.,* 1960, v79, 574.
19. Smith, C. G., Lummis, W. L., Grady, J. E., *Cancer Research,* 1959, v19, 847.

Received July 5, 1960. P.S.E.B.M., 1960, v105.

Effect of X-Radiation on Uptake of P^{32} into Individual Nucleotides of HeLa Cells.* (25993)

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Separation of the acid soluble phosphorus compounds of transplanted mouse mammary carcinoma and determination of their relative specific activities 2 hours after injection of radioactive phosphate have been described (1). It was demonstrated that 4500 r (in air) localized to the tumor brought about a lowering of relative specific activities of some polyphosphonucleotides. Because these tumors contained a variety of cells, it was thought that the results obtained might be due to changes in metabolism of cells other than those of the mammary carcinoma. To investigate the effect of non-malignant cells, the work was repeated employing Ehrlich ascites carcinoma grown in peritoneal cavities of susceptible mice. These cells can be isolated as a relatively homogenous line. The results of this study are being described elsewhere(2). As a further step in employment of a pure cell line, we have continued our studies using HeLa cells grown and irradiated *in vitro*. This latter investigation is the subject of this report.

Materials and methods. Cultures of HeLa cells(3) were propagated in Eagle's basal medium(4) supplemented with 10% human serum. Cells were harvested by rinsing with bicarbonate buffered glucose-KCl-NaCl solution followed by dispersal with 0.05% trypsin (Difco 1:250) as previously described(5). The trypsinized cells from about 100 bottles were pooled and centrifuged at 200 g for 10 minutes. Cells were resuspended in 25 ml of 10% human serum, 90% Hanks' balanced salt solution and enumerated. The suspension was divided and one-half containing at least 10^8 cells was subjected to irradiation. The bottle containing the cells was placed on its side on pressed wood backing material. The surface of the medium was 35 cm from the focal spot of the x-ray tube. The x-ray machine was operated at 220 KP, 15 MA with no filter (HVL 0.35 mm Cu). A total dose of 5000 r including backscatter was delivered at surface of medium. Cells were then incubated for 2 hours at 37°C, 4 ml of a solution of disodium hydrogen phosphate containing 200 μc of P^{32} having been added to each 30 ml of suspension. After centrifugation 200 g for 10 minutes, supernate was discarded. The cells were resuspended in 20 ml of Hanks' balanced salt solution and recentrifuged. The washed cells were homogenized with 4 volumes of ice cold 0.6 N perchloric acid in a glass Potter-Elvehjem homogenizer. After refrigeration for 30 minutes at 4°C, the homogenate was centrifuged in the cold at 1300 g for 30 minutes. Supernate was decanted off, and precipitate washed with 5 ml of 0.2 N perchloric acid. The combined extract and the washings were neutralized with cold 5 N potassium hydroxide and refrigerated overnight. After removal of potassium perchlorate by centrifugation, aliquots were taken for determination of radioactivity, total phosphorus(6), and nitrogen(7). The neutralized extracts were placed on twin columns of Dowex 1 (formate) X 8, 200-400

* This investigation supported by grants from U. S. Public Health Service, Am. Cancer Soc., Minnesota Division, and Am. Cancer Soc.