In vit. E-deficient, but not in starved animals, a significant increase in inulin and sucrose space of skeletal muscle was found. Whether this increase reflected an actual increase of the extracellular fluid compartment or was caused by increased permeability to the saccharides, could not be established. It was concluded that if there was an actual increase of extracellular space in the dystrophic muscle, this increase was not greater than by a factor of 2. Inulin space in livers of all 3 experimental groups was very variable and in most cases much higher than average sucrose space.

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Mammalian Cell Cultures Contaminated with PPLO III. Elimination of PPLO with Specific Antiserum.* (27985)

MARY E. POLLOCK AND GEORGE E. KENNY† (Introduced by L. C. McLaren)
Department of Microbiology, University of Minnesota, Minneapolis

The discovery in 1956 by Robinson and coworkers that mycoplasma species (pleuropneumonia-like organisms or PPLO) occurred as contaminants of mammalian cell cultures (1) stimulated further investigations of the extent of the problem, and led to a search for means of control which would not incur damage to cells of host cultures. Some broad-spectrum antibiotics have proven effective(2, 3); although previous reports indicated that doses toxic to mammalian cells were necessary, Carski and Shepard have reported elimination of PPLO from cell lines cultivated through 4 successive passages in the presence of only 2.5 μg of tetracycline per ml of medium(3). In this laboratory it was demonstrated that PPLO could be eliminated consistently from monolayer mammalian cell cultures by treatment with non-toxic levels of kanamycin when care was taken to use young, sparsely populated cultures and to allow contact with the antibiotic on all surfaces of the vessels(4). Even though antibiotics may be used simply and effectively, emergence of resistant strains must be anticipated whenever large numbers of microorganisms are concerned, and it is readily apparent that some alternative procedure should be available. With this in mind a study has been carried out to determine whether antiserum specific for the PPLO contaminant of a cell line would, when incorporated into the culture medium, cause eradication of the PPLO. Added impetus was given to this effort when a suspension of cells submitted from another laboratory was found to

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† Present address: Dept. of Preventive Medicine, Univ. of Washington, Seattle.

TABLE I. Description of PPLO-Contaminated Mammalian Cell Lines.

Cell line	Source	Reference	Growth medium*	Sensitive to kanamycin
HE	Subline of HeLa (human cervical carcinoma)	7	HS20 YEM	+
KE	*idem.*	7	" "	—
LL	Normal human liver	8	" "	+
D_1b_1	Clonal line from human liver strain	9, 10	" "	+
HH100	Subline of HeLa adapted to growth in medium containing horse serum	7, 11	HoS40 BSS	+
EE	Clonal line from human tracheo-esophageal fistula	8, 10	HS10 BME	+

* HS = human serum; HoS = horse serum. Numeral gives % of total volume. BSS = Hanks' balanced salt solution(12). YEM = BSS containing 0.1% yeast extract (Difco). BME = basal medium of Eagle as described previously(5).

be contaminated with a mycoplasma strain resistant not only to kanamycin but to all other antibiotics and chemotherapeutic agents tested. In this case, kanamycin-resistance was not the result of exposure to the antibiotic, but nevertheless made necessary the application of an alternative approach for elimination of the contaminant.

Materials and general methods. Mammalian cell lines. Pertinent data concerning the PPLO-contaminated cell lines employed are given in Table I. The corresponding mycoplasma strains are designated by the cell line code preceded by 'PPLO'.

The method for propagation and periodic transfer of stock monolayer cultures to clean vessels has been described(5,6). Cultures were incubated at 37°C and fed twice weekly by complete medium replacement.

For experimental purposes the growth media listed were altered by replacing a given amount of the serum routinely employed, with normal rabbit serum or immune serum specific for the mycoplasma strain present in the cell line. Before use all sera were inactivated at 56°C for 30 minutes unless otherwise specified.

Experimental procedures. Mammalian cell cultures were established in 16 × 125 mm screwcap tubes. After 24 hours' incubation growth medium was removed and replaced with test medium containing specific anti-PPLO or normal rabbit serum. Parallel PPLO-free controls of all except the KE line (kanamycin-resistant PPLO) were obtained by addition of 100 μg kanamycin‡ per ml of medium(4). Cultures were not again fed during the initial treatment period unless specified, but once daily the tubes were inverted and rolled to allow the fluid to contact all inner surfaces. After the designated period of exposure to test serum those cultures which had not been used for cell enumeration were fed routinely with control medium and tested periodically to determine whether PPLO were still present.

Cell counts were performed according to the procedure described earlier, using a Coulter electronic counter, model A§(5).

The method for *isolation of PPLO* was the same as that previously described(4) except that a medium containing soy peptone powder,‖ more recently introduced in this laboratory(6,13), replaced the pancreatic digest of beef heart formerly employed. The basal medium was supplemented with 15% filtered human serum. Viable counts were obtained by the method of colony enumeration previously given in detail(5). Cell cultures were tested for PPLO during the period of treatment with specific anti-PPLO serum, but negative results were considered significant only after extended periods of cultivation in medium containing normal serum.

Antigen for *preparation of antiserum* was obtained as follows. An agar block containing a single colony was transferred to broth medium, and subcultures were made daily for 5 days. The final 24-hour culture was then stored at −20°C in small aliquots which served as inoculum for preparation of antigen. For immediate use 2 to 3 hundred ml of 24-hour

‡ Kanamycin sulfate was kindly supplied by Bristol Laboratories, Inc., Syracuse, N. Y.

§ Coulter Electronics, Inc., Chicago, Ill.

‖ Obtained through the courtesy of Sheffield Chemical, Division of Nat. Dairy Products Corp., Norwich, N. Y.

broth culture was centrifuged at 8000 × *g* for 30 minutes, and the organisms were washed twice with equal volumes of GKNP (Hanks' balanced salt solution lacking calcium and magnesium salts and adjusted to pH 8.0 with sodium bicarbonate). The final pellet was resuspended in a volume one-tenth that of the original culture. The entire procedure was carried out at 2-4°C, and viable count after concentration was 50 to 70% of that prior to washing. Suspensions were used immediately for immunization of Dutch Belt rabbits; each animal was given 4 injections intraperitoneally at 4-day intervals. The inoculum ranged from an initial dose of 10^7 colony-forming units to about 10^8 in the final injection. Blood was removed aseptically by cardiac puncture 7 days after the last injection, and, unless otherwise stated, was allowed to clot at room temperature for 14 to 16 hours before removal of serum. The serum was stored at −20°C and was thawed just before use and inactivated at 56°C for 30 minutes if desired. It was shown that the antisera agglutinated PPLO of the homologous strain at a dilution of 1 in 320 or greater.

Results. Elimination of PPLO by use of inactivated specific antisera. Suspensions of 4 mammalian cell lines (HE, LL, D_1b_1, and HH100) were allowed to establish in growth medium (Table I) containing 5% antiserum specific for the PPLO in question. In accordance with reports that complement was not necessary for destruction of PPLO(14) all sera were inactivated. Five days after establishment the cultures were fed with the same medium, and 6 days later with control medium; total period of treatment was 11 days. Cultures for PPLO, first performed 5 days after the first feeding with control medium, were negative and remained so until the experiment was terminated 5 months later, although cultures of all 4 lines were reestablished repeatedly during that period. Parallel untreated controls continued to reveal PPLO consistently.

Effect of elimination of PPLO upon mammalian cell growth. Previous studies had shown that under certain conditions elimination of PPLO by addition of kanamycin to growth medium was accompanied by stimulation of the growth rate of mammalian cells(5). It was of interest to determine whether the growth rate of recently established PPLO-contaminated cell cultures could be enhanced by addition of antiserum specific for the PPLO contaminant. For this purpose test cultures were established in control growth medium. Twenty-four hours later the standard medium was removed and replaced with test fluid containing 5% anti-PPLO serum (HS15 IRS5 YEM80) or 5% normal rabbit serum (HS15 NRS5 YEM80). Parallel cultures were cultivated in the latter medium containing 100 μg kanamycin per ml. All sera had been inactivated at 56°C for 30 minutes. Four replicate cultures were employed at 2-day intervals for mammalian cell enumeration. Cells were not fed during the experimental period, but representative treated cultures, held longer and fed with control medium, were repeatedly reestablished and tested for PPLO. The organisms were not isolated from such cultures at any time during the period of observation, approximately 6 months.

Fig. 1 shows the relative number of HE cells in cultures containing 5% anti-PPLO-HE serum as compared with results in presence of normal rabbit serum. On the eleventh day the population of cultures grown in medium containing immune serum was 4-fold that of those cultivated in normal rabbit serum and equal to that of cultures grown in the presence of kanamycin. The populations of cultures propagated in HS20 YEM80 (not plotted here) were identical to those grown in medium containing normal rabbit serum.

A similar experiment with a slowly growing cell line, LL, failed to show stimulation of cell growth during 6 days' observation of cultures grown in medium containing anti-PPLO-LL serum. However, PPLO were never again isolated from cultures treated for a period of 10 days.

The populations attained by cultures of a second subline of HeLa (KE) in the presence of specific anti-PPLO serum as compared with those cultivated in medium containing normal rabbit serum are plotted in Fig. 2. A marked stimulatory effect upon growth was apparent when the PPLO were inactivated. In this case, in contrast to the previous experiments, it was necessary to use unheated serum to effect elimination of the PPLO; the contami-

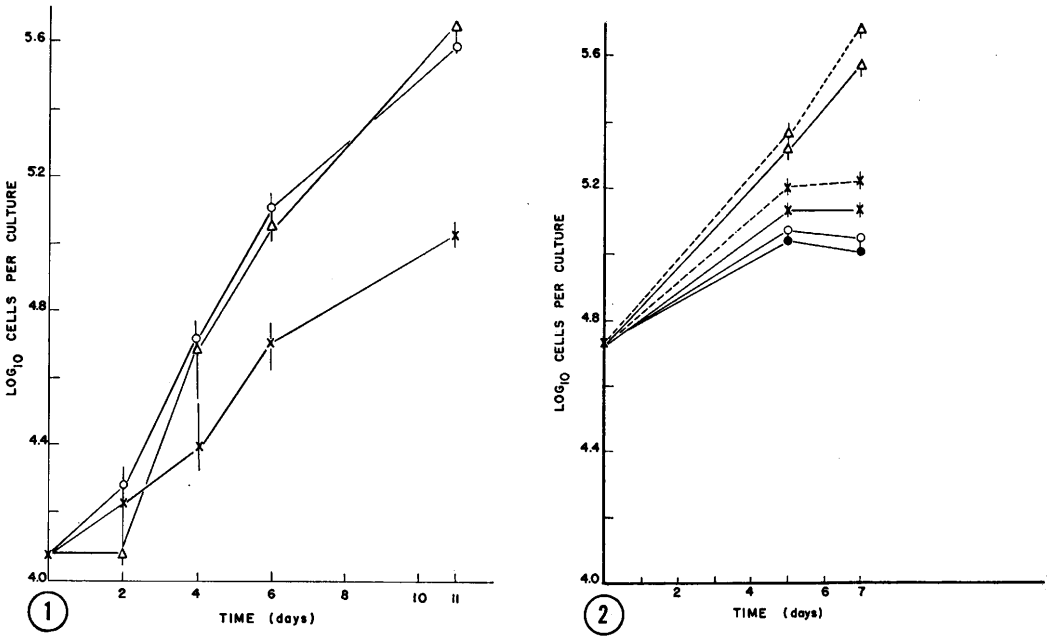

FIG. 1. Effect of specific inactivated anti-PPLO-HE rabbit serum on growth of PPLO-contaminated HE cells. After 24 hr incubation in HS20 YEM80, test media were applied; 25% of serum content of growth medium replaced with: ×——×, normal rabbit serum; ○——○, normal rabbit serum with 100 μg kanamycin added per ml final medium; △——△, anti-PPLO-HE rabbit serum. Vertical lines represent range of values obtained from 4 replicate cultures.

FIG. 2. Effect of specific unheated anti-PPLO-KE rabbit serum on growth of PPLO-contaminated KE cells. After 24 hr incubation in HS20 YEM80, test media were applied; HS20 YEM80 control, ○——○; control with 100 μg kanamycin added per ml final medium, ●——●; 25% of serum content of medium replaced with normal rabbit serum, ×——×, or anti-PPLO-KE serum, △——△; 50% of serum content of medium replaced with normal rabbit serum, ×---×, or anti-PPLO-KE serum, △---△. Vertical lines represent range of values obtained from 4 replicate cultures.

nants continued to grow when inactivated serum was used under the same conditions. This was also true of the strain of PPLO carried by the EE line.

Significance of exposure time in permanent elimination of PPLO. The preceding experiments demonstrated that PPLO could be permanently eliminated from cell culture with anti-PPLO serum but did not indicate the minimal time necessary. Therefore, an experiment was designed to determine how long the period of treatment must be for permanent eradication. Replicate cultures of KE cells, established as described, were cultivated in medium containing 10% immune serum (HS10 IRS10 YEM80). At designated intervals the test medium was removed from cultures taken at random. They were then rinsed 4 times with GKNP, and control medium was added. At least once daily during the experimental period those cultures still containing test medium were inverted and rolled gently to distribute the medium over the entire surface of the vessels, in order that no viable organisms would escape exposure to antiserum. The cell culture fluids removed at the time treatment was discontinued were cultured for PPLO, and succeeding discard fluids from the same cultures were tested regularly unless the given culture became PPLO-positive. Table II gives the results of tests for PPLO performed on cell cultures treated with 10% anti-PPLO-KE serum either unheated or inactivated at 56°C for 30 minutes. Period of exposure ranged from 16 hours to 8 days. Using unheated serum it was possible to completely eliminate the PPLO from random cultures in 4 days, but consistent elimination required 6 to 7 days. Heated antiserum reduced the PPLO colony count, probably because of agglutination, but it had no apparent destructive effect. Inactivated hyperimmune serum

TABLE II. Results of Tests for PPLO on KE Cell Cultures Exposed for Varying Periods of Time to Medium Containing Anti-PPLO-KE Rabbit Serum.

Anti-PPLO serum	Duration of treatment, days 2/3	1	2	3	4	5	6	7	8
Inactivated									
#1*	0†	0	0	0	0	0	0	0	0
#2*	0	0	0	0	0	0	0	0	0
Not inactivated									
#1	0	0	0	0	1	1	1	2	2
#2	0	0	0	0	0	1	2	2	2

* Serum lots 1 and 2 were obtained from the same animal but differed in that lot 2 was drawn after hyperimmunization with one additional injection of antigen and was removed from the clot and used within one hour of cardiac puncture.

† Number of cultures negative for PPLO (cultures reported negative only if they remained so after repeated testing for a period of 3 months after treatment was discontinued); each value represents results of examination of duplicate cultures.

from the same animal, obtained after one additional injection of antigen 10 weeks after the first series, was no more effective than that obtained initially.

Discussion. Mycoplasma species have long been known for their resistance to certain antibiotics and chemotherapeutic agents, particularly penicillin(15). In early studies carried out in this laboratory it was found that mycoplasma from mammalian cell lines were similar in their lack of sensitivity to streptomycin, penicillin and sulfonamides; however, kanamycin proved effective against all mycoplasma species then under investigation and had no apparent effect upon the mammalian cells themselves(4). The ease with which antibiotics may be applied in cell cultures makes their use the method of choice for elimination of PPLO under usual conditions. However, these substances may be contraindicated if it is necessary to employ doses toxic for mammalian cells to eradicate the contaminants. The problem of antibiotic resistance also requires the use of a different approach to control of PPLO. Although kanamycin-resistant strains have not emerged as the result of the use of the antibiotic in this laboratory, it is possible that such mutants will appear if the antibiotic is employed routinely for large populations of cells. For this reason it seemed pertinent that alternative means for elimination of PPLO from cell cultures be sought; with this in mind we returned to the program of preparation of specific anti-PPLO serum which was first begun in 1958. This procedure, although a laborious one, has been successful in all cell lines to which it has been applied. The fact that one of these lines, obtained from another laboratory, carried a PPLO resistant to all antibiotics and chemotherapeutic agents tested would indicate that such an alternative may be useful when other methods fail.

We did not test the effect of heterologous anti-PPLO sera for elimination of PPLO. However, recent evidence indicates that antigenic similarity exists among many mycoplasmal strains from cell culture(16,17). Therefore, it is possible that antisera against a single strain or several strains might be useful in eradication of PPLO from diverse mammalian cell lines.

The growth rate of some cell lines was increased when inactivated specific anti-PPLO serum was present in the medium. This effect was similar to that demonstrated previously (5), when PPLO were eliminated by use of the antibiotic, kanamycin.

Permanent elimination of mycoplasma from 4 cell lines was readily accomplished by use of heat-inactivated antisera, but only unheated sera were effective against the PPLO in the EE and KE lines. The nature of the heat-labile component which participates in destruction of these PPLO strains is not known. However, results of preliminary studies indicate that it may not be possible to replace the factor with guinea pig complement. All antisera tested, whether heated or not, were capable of agglutinating organisms of the homologous strain.

The fact that PPLO are permanently eliminated from mammalian cell cultures propagated for relatively short periods in presence of specific anti-PPLO serum suggests that the major site of PPLO is extracellular, where the organisms are accessible to action of antibody. It follows then that PPLO occurring within the living cells, generally considered impermeable to antibody, may be incapable of multiplication.

Constant surveillance for the presence of PPLO is of utmost importance in the use of mammalian cell cultures as experimental sys-

tems. It is equally important that dependable methods of elimination be available. Little is known of the mechanisms by which these contaminants may alter the results of physiological and virological studies using mammalian cells *in vitro,* but there is mounting evidence that effects do occur(5,6,18,19), and it behooves the investigator to use every means at his command to control the problem.

Summary. A procedure for control of PPLO contamination in mammalian cell cultures has been presented as an alternative to the employment of antibiotics. Six cell lines have been freed of PPLO by comparatively short-term exposure to antiserum specific for the contaminant in question. For destruction of 2 of the mycoplasma species a heat-labile serum component was required; the remaining 4 lines were successfully treated with inactivated serum. Because control of these contaminants is of utmost importance in the use of cell cultures as experimental systems application of this technic may be advantageous when antibiotic resistance is encountered.

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Tachyphylaxis to Epinephrine and Its Modification by Cocaine.* (27986)

MARIO PÉREZ-REYES AND MORRIS A. LIPTON

Department of Psychiatry, University of North Carolina School of Medicine, Chapel Hill

Seevers and Woods(1), reviewing the phenomena of tolerance to pharmacological agents concluded that "no tachyphylaxis is developed to the pressor effects of epinephrine in the dog." This conclusion was based largely on the experimental findings of Winder *et al.*(2) and Essex(3). We have found no later evidence in the literature to alter the conclusions of Seevers and Woods, that tachyphylaxis to the pressor effects of epinephrine cannot be established. On the other hand Rosenthal and Di Palma(4) have demonstrated the development of tachyphylaxis to norepinephrine in the dog. Their findings stimulated us to investigate the possibility of producing acute tolerance to epinephrine.

In the experiments to be reported we have studied: (a) the experimental conditions under which tachyphylaxis to epinephrine can

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