TISSUE CELL CULTURE CONTAMINATION IN RELATION TO BACTERIAL PLEUROPNEUMONIA-LIKE ORGANISMS-L FORM CONVERSION

NELDA B. HOLMGREN[1] AND WILLIAM E. CAMPBELL, JR.

Fort Detrick, Frederick, Maryland

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The source of the widespread infection of tissue cells with pleuropneumonia-like organisms (PPLO) has not been established. A number of potential sources of infection have been postulated, among them the formation of L phase variants (stabilized L forms) induced by the action of penicillin on contaminating bacteria (Rothblat and Morton, 1959). This paper describes the experimental induction of an L phase variant from a gram-negative bacillary contaminant of a tissue cell culture (NCTC clone 929, mouse fibroblast) when treated with polymyxin B and the subsequent reversible conversion of the induced "stable L form" to the original bacterial form.

MATERIALS AND METHODS

The original culture of mouse fibroblast cells (B 929-345-302) had been maintained without antibiotics by Dr. Earle at the National Institutes of Health for 345 generations and was demonstrated to be free of infection on receipt in our laboratory.

The media employed for the isolation of the L forms were heart infusion agar (blood agar base, Difco) and heart infusion broth (Difco) prepared according to the directions. The formula containing tryptose was used; pH was not adjusted. After autoclaving for 15 min at 121 C, the agar medium was cooled to 56 C and 20 per cent by volume of horse serum (Microbiological Associates), inactivated at 56 C for 30 min, was added. The pH of the medium after addition of the serum was approximately 7.4. The medium was poured into 50-mm plastic petri plates, 5 ml per plate. These plates have a tight fitting cover which helped to maintain a suitable humidity. The heart infusion broth was used with or without 20 per cent horse serum. Where yeast extract (Difco) was employed, it was added in a 1 per cent concentration to the agar or broth. Thioglycolate broth (Difco, without glucose) was also used for sterility tests.

Cultures for the L forms were made by inoculating 0.1 ml of culture or supernatant tissue culture medium containing a few tissue cells, scraped from the bottle with a bent-tip glass pipette, into one or more heart infusion serum broth tubes. The tubes were incubated aerobically at 37 C for 48 hr. Following incubation, 0.1 ml of the broth was subcultured to the surface of a heart infusion serum agar plate, rotated gently to spread the inoculum and incubated aerobically in an inverted position at 37 C. The plates were examined microscopically with the low power objective through the agar layer, daily for 6 days, before discarding the cultures as negative for L forms. Wet stains of the colonies were made by the method of Dienes (1942).

Antibiotic therapy of the tissue cells consisted of adding the designated units or μg of the antibiotics to the culture medium at the time of subculture and whenever medium was renewed.

RESULTS

The history of the culture is presented in figure 1. The original mouse fibroblast cell culture was propagated without antibiotics for approximately 6 weeks during which the cells were subcultured four times with intervening medium renewals at 2- to 3-day intervals. Tests for PPLO and other contaminating microorganisms, performed each time the tissue cells were subcultured, were negative.

After 6 weeks, the culture became contaminated with a gram-negative rod. Since the use of penicillin at this time was being avoided as a possible agent for inducing L forms, the cultures were treated with 200 μg per ml of chlortetracycline and 100 μg per ml of streptomycin. A bacteriostatic effect was noted but the infection with the bacillary form persisted. Therapy with 25 units

[1] Present address: Microbiology Department, Presbyterian-St. Luke's Hospital, Chicago, Illinois.

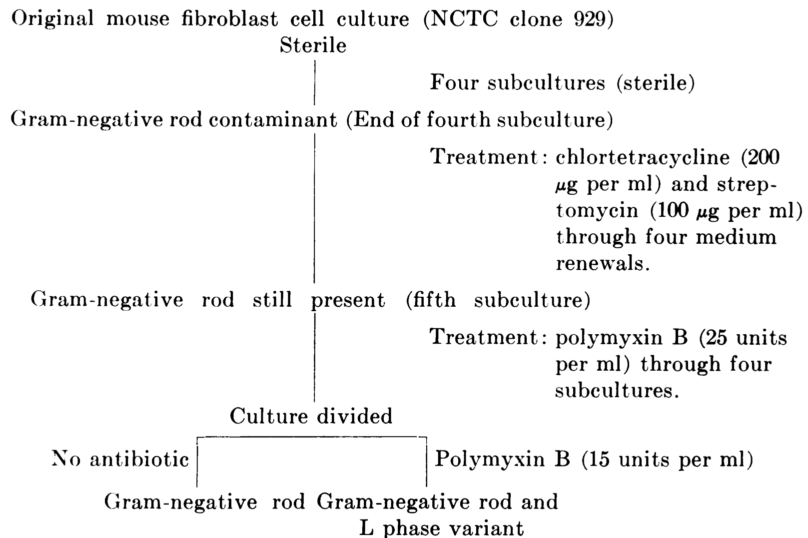

Figure 1. History of the mouse fibroblast cell (NCTC clone 929) culture

TABLE 1
Bacterial conversion to an L phase variant induced by polymyxin B

Conc. of Polymyxin B in Broth*	Type of Growth on Agar† Seeded from 24-hr Broth Culture
units/ml	
0	Gram-negative rod
5	L phase variants
15	L phase variants
25	L phase variants
50	L phase variants‡
100	L phase variants‡
1000	No growth

* Heart infusion broth plus 20 per cent horse serum.

† Heart infusion agar plus 20 per cent horse serum.

‡ Colonies appeared smaller when plated from these concentrations of polymyxin B.

per ml of polymyxin B was instituted for a period covering four subcultures and the intervening medium renewals. The culture was then divided into two sublines, one of which was carried without antibiotics and the other continued on polymyxin B (15 units per ml). From the former a gram-negative rod was isolated, and from the latter both the bacillary form and an L form were isolated.

A single colony of the bacillary form was isolated, cloned by subculture, and inoculated into heart infusion broth containing 20 per cent horse serum and varying concentration of polymyxin B, from 0 to 1000 units per ml. Following incubation for 24 hr at 37 C, 0.1 ml of each was plated on heart infusion agar supplemented with 20 per cent horse serum. The results are presented in table 1. L forms grew on the plates seeded from the broth cultures containing 5 to 100 units of polymyxin B per ml, whereas no growth occurred in cultures exposed to 1000 units. Only the bacillary form was recovered from the control broth tube containing no antibiotic.

The degree of exposure to polymyxin B required to commit the bacterial form to the L state was determined in the following manner. To check for the presence of L forms, the strain (357B) of the bacillary form used in this experiment was plated in dilutions of 1:50, 1:2500, and 1:12,500 on heart infusion agar, with and without 20 per cent horse serum, and on heart infusion serum agar containing 100 units per ml of polymyxin B. Only bacterial forms grew on all plates except those containing 100 units of polymyxin B, which showed no growth. The absence of growth on the agar plates containing polymyxin B is consistent with other results which followed a number of attempts to induce the conversion of the bacillary form to the L form in both surface and pour plate trials on solid medium containing polymyxin B. Reversible conversion of the bacterial and L form have been successful only in

liquid medium. Studies to determine whether agar concentration or tonicity of the solid medium (Landman *et al.*, 1957) were responsible for the negative results are incomplete.

The bacillary strain (357B) was also inoculated into 2-fold dilutions (range 0.39 to 200 units per ml) of polymyxin B in heart infusion broth (Difco) containing 20 per cent horse serum. At 24-hr intervals for a period of 6 days, each culture was diluted 1:50 and 1:2500 and plated on heart infusion serum agar. The results indicated that both the concentration of polymyxin B and the length of time of exposure to the antibiotic were factors that determined the stability of the induced L form. At concentrations below 12.5 units per ml of polymyxin B, stability was dependent on both concentration and time of contact of the bacterial form with the antibiotic. Above 12.5 units per ml, a stable L form was obtained in 24 hr following exposure of the bacterial form to the antibiotic.

TABLE 2

Conversion of an L phase variant to bacterial form

Series	Additions to Broth* Inoculated with L Form		No.	Additions to Agar Plage† Seeded with 48-hr Broth Culture of L-Form		Growth on Agar Plate
	Serum	Yeast extract		Serum	Yeast extract	
	%	%		%	%	
I	0	0	1	0	0	None
			2	20	0	None
			3	0	1	None
			4	20	1	None
II	20	0	1	0	0	None
			2	20	0	L forms
			3	0	1	None
			4	20	1	None
III	0	1	1	0	0	None
			2	20	0	None
			3	0	1	None
			4	20	1	None
IV	20	1	1	0	0	Bacillary form
			2	20	0	Bacillary form
			3	0	1	Bacillary form
			4	20	1	Bacillary form

* Heart infusion broth.

† Heart infusion agar.

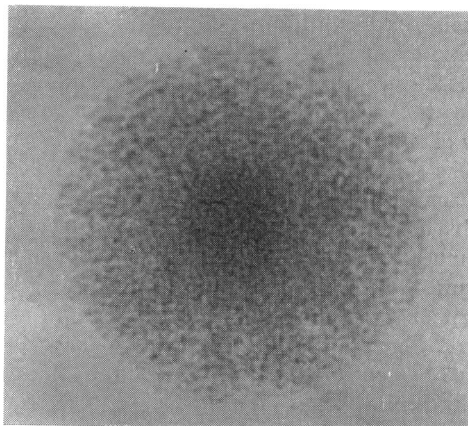

Figure 2. Colonial morphology of L phase variant. Wet methylene blue stain (magnification, ×228).

The cloned L form proved to be stable and attempts to convert it to the bacillary form were unsuccessful until 1 per cent yeast extract was added to the heart infusion serum broth. The results of a typical experiment are shown in table 2. The purified L phase variant was inoculated into heart infusion broth with or without the addition of either 20 per cent serum, 1 per cent yeast extract, or a combination of similar concentrations of serum and yeast extract. These cultures were incubated for 48 hr at 37 C. Following incubation, each culture was inoculated on heart infusion agar plates with or without the same additives.

The requirement of the L phase variant for serum explains the lack of growth in series I. L phase variants were obtained only when both the broth and the agar contained horse serum (series II, 2). In contrast, no growth was obtained in series II, 4, although horse serum was present in both the broth and agar. Series II, 4, differed from series II, 2, however, in that 1 per cent yeast extract was present in the agar. On the other hand, when 1 per cent yeast extract was added both to the serum broth and to the agar, with or without serum (series IV), it was differentially selective for the growth of the bacterial forms, possibly by supplying to the L phase variants a thermostable factor essential for cell wall synthesis.

At no time after purification and isolation of each form from the tissue culture were both forms simultaneously found in cultures of either the L phase variant or the bacillary form. It is almost

certain that both forms were not present in the L form inoculum used in the experiment shown in table 2, since bacillary forms were not obtained in series I, II, and III; the bacillary forms grow in any of these media.

The L form requires serum for growth in heart infusion broth or soft heart infusion agar and is stable on subculture in the absence or presence of as much as 200 units of polymyxin B per ml. Figures 2 and 3 are photographs illustrating the morphology of a wet-stained colony on an agar block. Figure 4 shows an impression smear prepared by gently lifting the cover slip from a wet-stained agar block preparation. Growth, composed of pleomorphic minute bodies, extends centrally into the agar.

On primary isolation from the tissue culture, colonial and bacillary morphology were very pleomorphic. The organism grew slowly and required the presence of serum. On cultivation in laboratory media, this pleomorphism was lost and the bacillary form grew in the absence of serum at both room temperature and 37 C. Recent findings indicate that selection against the reversible conversion occurs when the L phase variant and the bacillary form are cultivated in cell-free laboratory medium. Studies are continuing to determine the reason for the observed increased selection and stability of these forms.

The gram-negative rod has not been identified. It is aerobic, motile at room temperature, does not ferment glucose, and produces a diffusible yellowish-brown pigment which shows a green fluorescence in ultraviolet light.

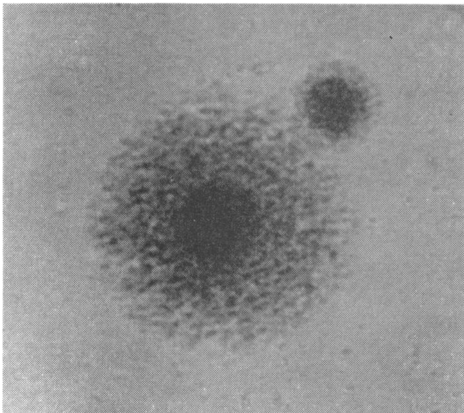

Figure 3. Colonial morphology of L phase variant. Wet methylene blue stain. Photographed with green filter (magnification, ×228).

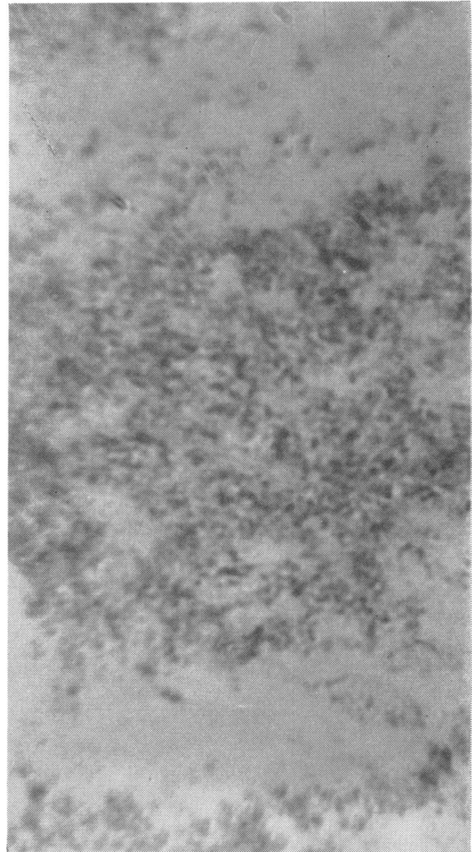

Figure 4. Impression smear of an L phase variant culture. Methylene blue stain (magnification, ×1180).

DISCUSSION

The experiments described in this report support the hypothesis that the widespread infection in tissue cultures with pleuropneumonia-like organisms results from the common use of antibiotics to control bacterial contamination. The history of the tissue cell infection indicates clearly the possible role of antibiotics in the conversion of bacterial contaminants to stable L phase variants. Furthermore, the bacterial isolate from the tissue cells could be taken through complete cycles in cell-free laboratory medium to a stable L form and from these derived L forms back to the original bacterial form. Based on this hypothesis of the source of infection, tissue cultures have been maintained without antibiotics for a period of nine months and have remained free of PPLO infection.

After a period of subculture in laboratory

medium, differences in morphology and growth of the bacterial form as well as selection against conversion were noted. This raises the question whether the ability to produce stable L forms in the presence of polymyxin B is a property of certain variant cells in the bacterial population. It is tempting to speculate that environmental conditions in the tissue culture coupled with antibiotic treatment were favorable for selection of a variant susceptible to induction by polymyxin B. Since horse serum was present in the tissue culture medium as well as in the cell-free laboratory medium, it seems unlikely that a component of the serum was selective or responsible for the conversion.

Supportive evidence for the growth inhibitory effect of yeast extract shown in table 2, series II, 4, on the viability and growth of stable L forms on solid medium was presented by Edward (1950) who showed that yeast extract in horse serum agar plates either enhanced or inhibited the growth of certain strains of PPLO. Medill and O'Kane (1954) reported that yeast extract inhibited the growth of proteus L forms in agar medium but stimulated that of rod forms. They believed that L forms were sensitive to inhibitors in natural products and that the function of serum was one of detoxification. In the experiments reported here, yeast extract in liquid medium containing horse serum appeared to favor the reversion of L forms to bacterial forms whereas when present in similar solid medium, no reversion occurred and the L forms did not survive. On the other hand, Wittler *et al.* (1956) reported that yeast extract in concentrations of 0.5 to 4 per cent caused increased growth of PPLO in tissue cells and a change in colonial form, from a PPLO to an L form, subsequent to isolation from the tissue cells. Kawatomari (1958) was able to convert L colonies derived from a *Clostridium* species to the bacterial forms in brain heart infusion serum broth supplemented with 1 per cent yeast extract. In the light of our present knowledge, it is impossible to arrive at any satisfactory explanation for the variable response of PPLO and L forms to yeast extract.

Of interest is the antibiotic sensitivity pattern of the bacterial form and the L phase variant to polymyxin B. The bacterial form is sensitive and the L form resistant as indicated by its ability to grow in subculture in the presence of 200 units of polymyxin B. This resembles the sensitivity pattern of the parent and L forms induced by penicillin.

The mechanism of action of polymyxin B in inducing L forms is not altogether evident. The work of Newton (1956) indicated that polymyxin B combined with phospholipid components in the cell membrane resulting in disorganization of the cell membrane or osmotic barrier. However, the phospholipid components of the cell walls of gram-positive and gram-negative organisms have been found to differ in chemical composition and structure. Mitchell and Moyle (1954) reported that the content of lipid phosphorus in the gram-negative organisms was twice that in the gram-positive ones. Few (1954) observed a secondary layer or membrane closely adherent to the outer cell walls in gram-negative bacilli; no such structure was found in gram-positive organisms. In addition, Newton's (1956) results showed that 90 per cent of the polymyxin was bound by the protoplast membrane of gram-positive bacteria; in *Pseudomonas aeruginosa* the distribution was equally divided between the cell walls and the adherent membrane. Whether these differences in chemical composition and structure between gram-positive and gram-negative organisms can account for the ability of polymyxin B to induce stable L forms from a gram-negative rod is not presently known. One can speculate that in gram-negative organisms the polymyxin is bound by the phospholipids in the membrane adherent to the cell wall and that this, in some way, interferes with cell wall synthesis.

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SUMMARY

Conversion of a gram-negative rod to an L phase variant by polymyxin B and the subsequent reversion of the latter to the original bacterial form has been experimentally demonstrated both in tissue culture and in laboratory media after isolation of each morphological phase from the tissue culture. These results, together with the history of the tissue cell infection, support the hypothesis that the source of infections from pleuropneumonia-like organisms (PPLO) in tissue cells is the conversion of bacterial contaminants

to stable L forms by antibiotics commonly used to prevent bacterial contamination.

REFERENCES

DIENES, L. 1942 The significance of the large bodies and the development of L type of colonies in bacterial cultures. J. Bacteriol., **44,** 37–74.

EDWARD, D. G. 1950 An investigation of the biological properties of organisms of the pleuropneumonia group, with suggestions regarding the identification of strains. J. Gen. Microbiol., **4,** 311–329.

FEW, A. V. 1954 Electronmicroscopy of disrupted bacteria treated with polymyxin E. J. Gen. Microbiol., **10,** 304–308.

KAWATOMARI, T. 1958 Studies on the L-forms of *Clostridium perfringeus*. I. Relationship of colony morphology and reversibility. J. Bacteriol., **76,** 227–232.

LANDMAN, O. E., ALTENBERN, R. A., AND GINOZA, H. S. 1957 Quantitative conversion of cells and protoplasts of *Proteus mirabilis* and *Escherichia coli* to the L-form. J. Bacteriol. **75,** 567–576.

MEDILL, M. A. AND O'KANE, D. J. 1954 A synthetic medium for the L type colonies of Proteus. J. Bacteriol., **68,** 530–533.

MITCHELL, P. AND MOYLE, J. 1954 The Gram reaction and cell composition: nucleic acids and other phosphate fractions. J. Gen. Microbiol., **10,** 533–540.

NEWTON, B. A. 1956 The properties and mode of action of the polymyxins. Bacteriol. Revs., **20,** 14–27.

ROTHBLAT, G. H. AND MORTON, H. E. 1959 Detection and possible source of contaminating pleuropneumonia - like organisms (PPLO) in cultures of tissue cells. Proc. Soc. Exptl. Biol. Med., **100,** 87–90.

WITTLER, R. G., CARY, S. G., AND LINDBERG, R. B. 1956 Reversion of a pleuropneumonia-like organism to a Corynebacterium during tissue culture passage. J. Gen. Microbiol., **14,** 763–774.