Nucleoside Incorporation into Strain L Cells: Inhibition by Pleuropneumonia-Like Organisms

Abstract. *Contamination of Strain L cell cultures by pleuropneumonia-like organisms (PPLO) resulted in a complete inhibition of the incorporation of tritiated thymidine and uridine. Contaminated cultures were characterized by a concentration of PPLO along the margins of the cells and in the intercellular spaces of the cultures. Autoradiograms of PPLO-contaminated cultures were characterized by exposed silver grains over the margins of the cells. Kanamycin was effective in the elimination of PPLO and the restoration of nucleoside incorporation.*

Pleuropneumonia-like organisms—PPLO—are frequently found in mammalian cell cultures, especially when penicillin is used routinely and continuously to suppress the growth of bacteria (*1*, *2*). These organisms are usually found in the supernatant fluid but they may also exist as intracellular parasites (*3*). Contamination of tissue cultures by PPLO is a problem of increasing importance because their presence is often not readily manifested and the subtle consequences of their existence on the behavior of mammalian cells has yet to be thoroughly explored.

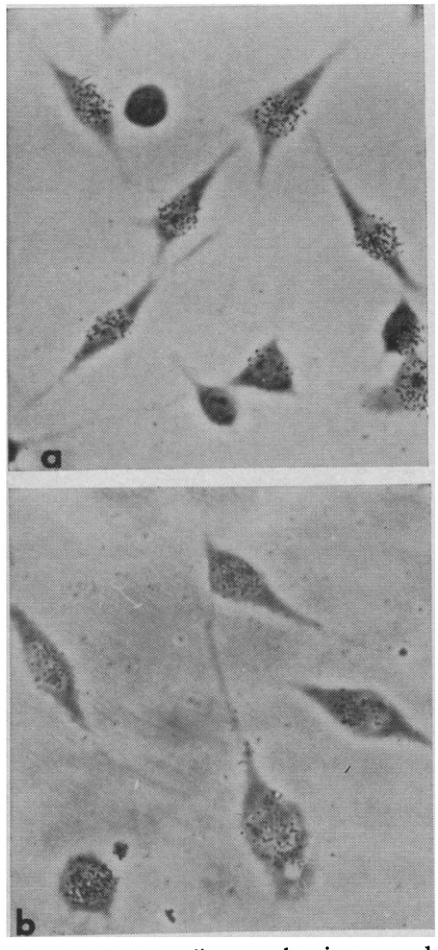

Fig. 1. (*a*) Autoradiogram showing normal pattern of tritiated-thymidine uptake by Strain L cells. (*b*) Normal pattern of tritiated-uridine uptake. (× 400)

It is well known that intracellular parasites, such as viruses, affect the metabolism of host cells (*4*) and that the arginine metabolism of PPLO contaminants in mammalian cell cultures may lead to erroneous conclusions (*5*), but we are unaware of any reports that deal with the effect of contamination by PPLO on the metabolism of mammalian cells, other than a reduction in growth rate as a result of arginine depletion (*6*). This is a report of a correlation between the contamination of Strain L cell cultures by PPLO and an inhibition of the incorporation of the nucleosides thymidine and uridine by the mammalian cells.

Stock cultures of cells were grown as monolayers in 4-ounce (125-ml) prescription bottles containing Eagle's minimum essential medium (MEM) (*7*) fortified with horse serum at a concentration of 5 percent. Autoradiograms of cells grown as monolayers on flying cover slips were prepared in Leighton tubes; standard procedures were used. The cells were harvested from stock bottles by trypsinization. Forty-eight hours after inoculation, medium containing tritiated thymidine (Schwarz BioResearch; 1.9 c/mmole) at a concentration of 0.20 μc/ml was incubated with duplicate cultures for 30 minutes; tritiated uridine (Schwarz BioResearch; 360 mc/mmole), at a concentration of 0.20 μc/ml was also incubated with replicate cultures for 30 minutes. After the cells were washed, fixed, and dried, they were coated with Eastman Kodak nuclear track emulsion NTB2. The thymidine series was exposed for 2 days and the uridine series for 8 days. Typical autoradiograms of uncontaminated cultures are shown in Fig. 1, *a* and *b*. The incubation time for the uridine series allows for nuclear label only.

During the 3 years since the inhibition of nucleoside uptake was first noticed, it has interfered with at least five different nucleic acid experiments in which isotopes and autoradiography were employed. Cells brought to this laboratory from other institutions were able to incorporate nucleic acid precursors for only 2 or 3 months before the inhibition of uptake occurred. Inhibition, once started, appeared to increase at a continuous rate for 1 to 3 weeks until complete inhibition was observed. This period was characterized by a continuous decline in the incorporation of thymidine and uridine into the nucleus and a concomitant increase in the appearance of isotope over the cytoplasm and along the margins of the cells (Fig. 2). During the period of decline in the rate of incorporation, the generation time (24 hours) remained unchanged and no cytopathological effects were seen. In one experiment the cells were incubated with tritiated thymidine every 2 hours for a period of 28 hours. These cells labeled normally for 6 hours, but at 8 hours a decline in nuclear label was observed and this continued until no isotope was found over the nucleus after 12 hours.

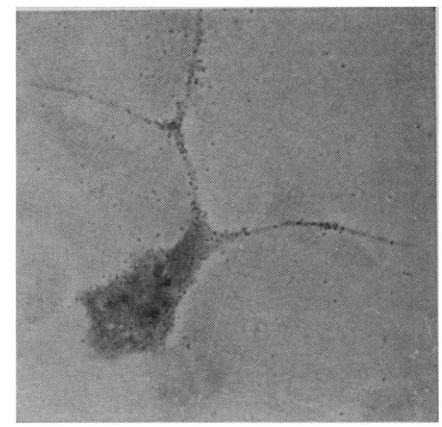

Fig. 2. Autoradiogram of a cell from a culture contaminated by PPLO, incubated in tritiated thymidine. It shows distribution of isotope along the cell margins and the absence of nuclear label. (× 400)

Tests for bacterial contamination were made by inoculation of cell-culture fluid into thioglycollate and trypticase soy broth. These tests were negative. However, incubation of cells or supernatant fluid with Difco PPLO broth containing crystal violet, or plating on PPLO agar, or both, revealed the presence of typical colonies of PPLO. Furthermore, Giemsa staining of the cultures revealed an increase in the amount of extracellular debris. Stainable bodies along the margins of the cells appeared when the cells were stained with aceto-orcein (Fig. 3*a*) according to the procedure of Fogh and Fogh; this is indicative of the presence of PPLO colonies (*8*).

That PPLO are responsible for the inhibition and that the inhibition is re-

versible were demonstrated with two sublines of Strain L. Contaminated cultures were grown for 48 hours in medium containing 400 μg of kanamycin per milliliter, then maintained in medium with 100 μg/ml. Cultures treated in this way showed a normal amount and pattern of uridine and thymidine incorporation into the nucleus and did not have extracellular debris and isotope distributed along the margins of the cytoplasm (Fig. 3*b*). Cultures decontaminated with kanamycin and able to incorporate the nucleosides were then reinfected by the addition of supernatant fluid taken from contaminated cultures. Inhibition of the incorporation of thymidine was manifested once again, while control cultures continued to incorporate the thymidine and uridine.

The final reinfection-type experiment was conducted in the following way. A contaminated culture (no nucleoside incorporation into nucleus) was split into two stocks, A and B. Stock A was treated with kanamycin, and normal incorporation of nucleoside took place. Stock B cells were concentrated by centrifugation and inoculated into PPLO broth. After 2 weeks, a portion of the PPLO broth was incubated with a kanamycin-treated culture for 24 hours, and then the medium was completely replaced with sterile MEM. Autoradiograms prepared 72 hours later showed no nucleoside label over the nuclei of cells of the reinfected cultures, while cultures of stock A, which were treated with sterile PPLO broth, had normal nucleoside incorporation.

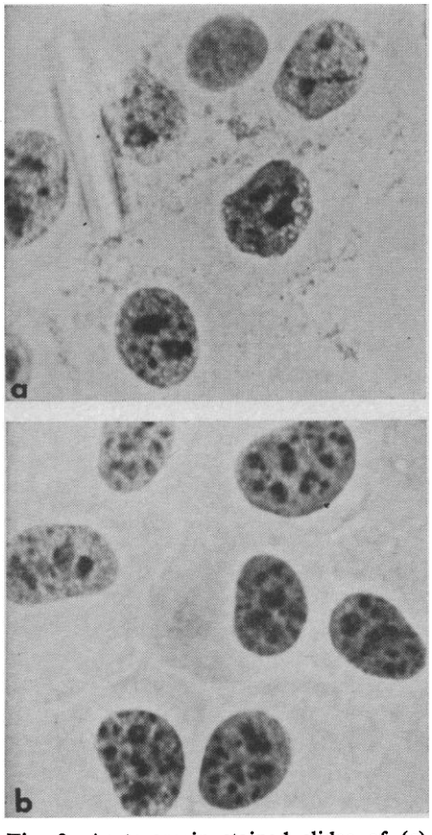

Fig. 3. Aceto-orcein stained slides of (*a*) a PPLO-contaminated culture of Strain L cells and (*b*) a culture of cells decontaminated with kanamycin. (× 1000)

Three experiments were performed to ascertain whether the lack of nucleoside incorporation stems from competitive inhibition. (i) Cells contaminated with PPLO were incubated in the tritiated thymidine for more than 24 hours (1 generation time) and the time for exposure of the emulsion was increased fourfold. No nuclear label was detected. The incubation time used was sufficient to allow all cells to go through the DNA synthesis (S) phase and was about 40 times longer than that usually used. (ii) Cells were also grown in medium containing tritiated thymidine at concentrations of 0.25, 0.50, and 1.0 μc per milliliter. This represents a two- and fourfold increase above that ordinarily used. Once again, no label was detected. (iii) Contaminated cultures were incubated in medium containing tritiated thymidine. No incorporation was observed. The supernatant fluid from these cultures was decanted under sterile conditions and added to cultures which had been treated with kanamycin. The cells of these cultures were typically labeled, thus demonstrating that the lack of label in the first culture was not due to depletion of the thymidine by the PPLO. Since no nuclear label whatsoever was detected under these conditions, competitive inhibition was ruled out as the cause of the phenomenon.

The use of labeled nucleosides in autoradiography as a measure of nucleic acid synthesis is based on the theory that cells will tend to conserve available energy and precursor sources and preferentially utilize preformed material when available. Since the cells continue to divide in spite of the inhibition of nucleoside uptake, one may assume that *de novo* DNA synthesis is adequate for normal growth. The PPLO must be effecting an inhibition which is not critical for cell growth. Two mechanisms are proposed. (i) The high arginine requirement of PPLO and the possible involvement of arginine in cell membrane transport suggests that if the PPLO are depleting the arginine supply normally available to the cells, transport can be adversely affected. (ii) In view of the fact that uridine and thymidine are incorporated into nucleic acids as triphosphates, suppression of phosphorylation enzymes looms as another possible explanation.

It is believed that the phenomenon reported here may be more widespread than the dearth of reports suggests because more than 50 percent of the cell lines currently in use may be contaminated by PPLO (*2*) and because cells from three different sources were used in this study. We have noted a profound alteration in the metabolic pattern as a result of contamination by at least one type of PPLO. Since much fundamental metabolic research with cell cultures was done at a time when penicillin was used and PPLO contamination was ignored, it behooves us to view the results of that period with some caution.

After this paper was submitted for publication we were informed of the work of Randall *et al.* (*Science*, this issue) who observed that PPLO contamination of HeLa and Strain L cultures rendered the DNA unstable but had little effect on the growth rate. These findings cannot account for the failure of our contaminated cultures to show any nucleoside incorporation. Indeed, if the uptake of nucleoside had not been inhibited at a time when the turnover rate was high we would have encountered labeling heavier than usual. Nevertheless, their findings do underscore the fact that contamination by PPLO has dramatic and possibly variable effects on cell metabolism.

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References and Notes

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