Establishment of Long-Term Cultures of Ehrlich Ascites Tumor Cells.* (27286)

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A simple method is presented for establishing long-term cultures of mouse Ehrlich ascites carcinoma in which a majority of cells retain their characteristic round shape. This line of cells has been in continuous culture for a year. Until recently, attempts to establish and maintain ascites tumors in culture have resulted in failure(1-3), in short-term cultures(4-6), and in cultures that failed to produce tumors when inoculated into mice (4,7). Animal ascites tumors recently reported as established in culture have been the 6C3HED mouse lymphosarcoma(8), the Yoshida and AH-130 rat hepatomas(9), TA_3 mammary carcinoma and Ehrlich(10), Krebs-2 and Ehrlich (Lettré)(11), ES hypotetraploid line of Klein(12) and CCRF Ehrlich(13).

The present investigation is part of a program to develop, *in vitro*, quantitative experimental approaches to cancer chemotherapy screening now possible with many micro-organisms.

Materials and method. ELD hyperdiploid Ehrlich ascites was obtained from Dr. T. S. Hauschka in 1955. Since then, the tumor has been maintained by weekly transfer in ICR/Ha Swiss mice, and has been neither frozen nor cloned. In preparation for establishing a culture, about 0.5 ml of fluid was removed aseptically from a mouse inoculated 5 days previously. The suspension was placed in a chilled test tube and was diluted with 10 cc of sterile medium: 60% Earle's salts(14), containing 0.65% lactalbumin hydrolysate,† 20% fetal calf serum,‡ 20% bovine amniotic fluid,§ and streptomycin at a final concentration of 50 μg/ml. After further dilution, cells were counted with a hemocytometer.

Into several flat-sided 8-oz. "gem oval" bottles, 3-5 × 10^6 cells were inoculated, and the culture was diluted with sterile medium to a total of 20 ml. Half of the medium was replaced after 24 hours, and at 48-hour intervals for 10 days, at which time growth on glass was apparent. Subsequently all of the used medium and detached cells were removed, and fresh medium was added every 2 days. After 21 days, sufficient growth occurred so that subcultures could be made by reinoculating into a new bottle the supernatant containing dividing cells. Attempts to remove the monolayer by scraping with a rubber policeman or by the use of 0.25% trypsin in Earle's basic salt solution destroyed the entire population until cultures were 50 days old, when either technic was satisfactory for removing cells. At present, growth occurs so that subcultures may be prepared weekly with 5 × 10^6 cells.

A modification of the squash technic of Hsu and Klatt(15) was used for determination of chromosome numbers. One gamma of colchicine per ml of fresh medium was added to an 8-oz. bottle of cells in the maximum growth phase. After 18 hours of incubation, the cells were scraped from the bottle and were transferred to a 15-ml centrifuge tube. After centrifugation for 6 minutes at 100 *g*, the supernatant was decanted, and the cells were exposed to a hypotonic salt solution consisting of 25% Earle's solution and 75% distilled water for 15 minutes, with occasional shaking. The tube was centrifuged slowly, and the liquid was decanted, leaving a few drops. The remaining drops were mixed with a 0.1-ml pipette with twice their volume of 0.5% aceto-orcein stain. After 5 minutes, a drop of the stained preparation was placed on a slide, squashed, and mounted. Chromosome studies of *in vivo* cells were done with

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† Difco Laboratories.

‡ Hyland Laboratories.

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cells from animals given colchicine intraperitoneally. In all studies, 100 exact counts were taken.

Results. In primary cultures, attachment of cells takes place within ½ hour after inoculation. The cells appear round, with few fibroblast cells dispersed among them. At 72 hours, very few cells remain attached; by the seventh day, however, small scattered colonies appear, that consist of round as well as fibroblast and epithial-like cell types. By 21 days, a complete monolayer is formed, with cell types intermixed, but with the largest number of cells still round. If growth is permitted to continue, additional layers of cells form, and a large number of cells may be seen floating in the medium. Staining with trypan blue or phenol red proves that these cells are alive and dividing. After weeks of subculturing, the cells will survive either mechanical or enzymatic removal from the glass surface. Prolific growth is demonstrated by the growth of cells on the side of the culture vessel as well as in suspension in the medium. Microscopic examination reveals a predominance of round, lymphocyte-like cells with equally dispersed fibroblast-like and epithelial-like cells, and clear spaces between cells.

Modified versions of the basic medium were not successful in establishing growth. Concentrations of 2 to 40% human ascites fluid in combination with varying concentrations of fetal calf, calf, or horse serum in Earle's medium or Earle's basic salts did not support growth. Decreasing the concentration of fetal calf serum or amniotic fluid in the basic medium of established cultures retarded growth after 10 days; in two weeks, a large number of multinucleated giant cells and cells with vacuoles were observed. Eagle's medium(16) in place of Earle's salts produced damage within 24 hours. Other media, such as Hsu's(17) with 20% fetal calf serum, resulted in cells with increased granulation after 4 days. The use of 199 medium (18) plus 5% calf serum caused the formation of clumps after 2½ weeks. Calf or horse serum substituted for fetal calf serum in the basic medium failed to maintain growth.

Pathogenicity of Ehrlich ascites tumor cells grown in tissue culture was proven by tumor induction in Swiss mice. The cultures, products of 1, 2, 3, 6, and 12 months of continuous growth, were given by intraperitoneal injection into groups of 6 mice. Cells were scraped from culture bottles, suspended in a small amount of saline, counted, and injected in varying quantities. For the first 3 intervals, as few as 6,000 cells produced tumor incidences of 100, 66, and 50%, respectively. Subsequently a minimum of 10^4 cells was required for a tumor incidence of 100% at 6 months. When 10^5, 7×10^5 and 2×10^6 cells from cultures 1 year old were injected into mice, tumor growth was observed in 1/8, 8/8, and 4/4, respectively. The resulting tumors were easily maintained by serial mouse passages and gave rise to typical mouse abdominal distension due to ascites formation in 18-20 days. After being frozen and thawed, a 3-month-old culture produced growth *in vitro* and subsequently typical ascites tumor in mice.

TABLE I. Chromosome Changes of ELD Ascites Carcinoma Cells *In Vitro* and *In Vivo*.

	Modal chromosome No.	% aneuploid
Stock *in vivo*	44–46	6
Tissue culture 9 wk	74–77	96
Reinfected at 9 wk	47	10
Tissue culture 3 mo	69–78	96
Reinfected at 3 mo	76–81	82
Tissue culture 6 mo	69–73	100
Reinfected at 6 mo	69–77	80
Tissue culture 12 mo	67–75	100
Reinfected at 12 mo	67–75	100

Chromosome studies were done at the time of mouse injection and upon evidence of tumor induction in mice (Table I). The *in vivo* stock tumor was proven to be hyperdiploid with a mode of 44-46 chromosomes (average 44.7) and about 6% aneuploid cells. With continuous subculturing the karyotype shifted to an increased aneuploidic chromosome pattern. For the first 9 weeks the modal chromosome number was in the high seventies (74-77). Subsequently, the number of chromosomes increased with the appearance of cells having 200 chromosomes ($\sim$ 5%) and the elimination of all normal hyperdiploid cells. Up to 3 months, reinfection into mice resulted

in selection of cells with a chromosome mode which again resembled that of stock *in vivo* ascites cells. Reinfection of mice with cells cultured 3 months or more, or continuous culturing of cells *in vitro* resulted in a population of cells which were either predominately or completely aneuploid. Return of cells from mice previously inoculated with cells cultured 3 months or longer, to *in vitro* conditions, produced the same effect. After the year-old *in vitro* ascites had been grown in mice and subsequently tissue cultured again for 2 weeks a chromosome mode of 69-75 was found.

Discussion. Cells of this Ehrlich ascites tumor became well adapted to continuous culture on a glass surface. New cultures were begun and maintained without application of CO_2 gas as required by the method of Ely and Gray or Deschner and Allen(4,11), and without subjecting the cells to a freezing procedure(10). Establishment and maintenance of the cell line required supplementation of the synthetic medium with fetal calf serum and amniotic fluid. In contrast to the results of Cailleau and Costa(12) and Ely and Gray (11), the use of other culture media or the substitution of calf or horse serum for fetal calf serum in the basic medium proved readily deleterious to the *in vitro* growth of the cells.

Selection for the growth of cells with a large aneuploid number of chromosomes occurs in cultures maintained *in vitro*. At times there appears to be a further selection of cells with increased numbers of chromosomes, until at 6 months only 20 counts could be used for the mode, and some of the remaining 80 cells counted were found to have up to 200 chromosomes. Further subculturing was not accompanied by any other significant chromosomal changes. This represents a considerable shift to larger numbers of chromosomes. There was a return to the original number after a short period of growth in mice; however, after 6 months of *in vitro* growth, not one cell bearing the original stem line could be found. The medium used in these experiments apparently is more suitable for cells having a chromosome number at least twice, if not more than that of the original cells taken from a mouse.

Summary. The ELD Ehrlich carcinoma has been established as a cell strain in tissue culture. Cells grow on a medium of Earle's salts supplemented with fetal calf serum and bovine amniotic fluid. The modal chromosome number was 69-73 for the first 6 months; it then shifted to 67-75. Inoculation of tissue-cultured cells at all intervals tested induced tumor formation in mice.

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