

# *In Vitro* Culture of the Krebs Ascites Carcinoma and the Ehrlich Ascites Carcinoma of Mice\*

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## SUMMARY

Cells of the Krebs ascites carcinoma and of the Ehrlich ascites carcinoma of mice have been established in cultures some of which are still thriving after 8 months. Chicken embryo extract was found to be toxic for the cells of those tumors. Cultures from single cells of the Krebs ascites carcinoma and of the Ehrlich ascites carcinoma have been established.

Several attempts in this laboratory in the last 2 or 3 years to culture the Krebs-2 ascites carcinoma of mice had failed. The maximum survival time of the cells in culture was about 10 days. The media used always contained chicken embryo extract (CEE). Later, during efforts to develop a culture medium and technic by which single cell cultures could readily be initiated for single-cell irradiation experiments, it was observed that CEE was toxic for mouse embryo skin cells in that it caused death of the cells when small numbers were in culture.

Other workers (1, 6, 9, 10), with one exception, who used CEE in their culture medium were unable to establish and maintain, except for short periods, cultures of the cells of either the Krebs ascites carcinoma or of the Ehrlich ascites carcinoma of mice. Hull (6) maintained a culture of the Krebs ascites carcinoma cells for 8 months and then lost the culture. There is no report that he succeeded in establishing additional cultures of those cells. Graff and McCarty, using a medium without CEE (4), obtained good growth of the Ehrlich ascites carcinoma cells in a cytogenerator for 15 days.

The present investigations have shown that CEE is toxic for the cells in culture of both the Krebs ascites carcinoma and the Ehrlich ascites carcinoma.

## MATERIALS AND METHODS

*Tumors.*—The Krebs-2 ascites carcinoma of mice has been maintained in Swiss mice since 1952, at which time the tumor was obtained from

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the Institute for Cancer Research and the Lankester Hospital Research Institute, Philadelphia, Pennsylvania. The Ehrlich (Lettré) ascites carcinoma was obtained in 1957 from the National Institute of Arthritis and Metabolic Diseases, Bethesda, Maryland, and maintained here in Swiss mice.

*Culture vessels.*—Pyrex glass culture vessels of the Petri dish type, 30 and 60 mm. in diameter, were used. The culture vessels, as well as other glassware, were cleaned by being boiled in water containing a small amount of Dreft, then thoroughly rinsed with tap water and distilled water. The culture vessels were sterilized by being dipped into absolute ethyl alcohol and then flamed to burn the alcohol.

*Media.*—Triple glass-distilled water was used in the media. Solutions that required sterilization were passed through a Selas filter #02.

Two types of media were used: (a) a combination of serum, balanced salt solution and CEE,<sup>1</sup> and (b) a semi-synthetic medium fortified with calf or horse serum. The medium No. 858 of Healy, Fisher, and Parker (5), somewhat modified, was used almost exclusively (Table 1).

Limited use was also made of the following media, NCTC 107 (2, 3),<sup>2</sup> NCTC 109 (2, 3),<sup>2</sup> Weymouth's No. 752/1 (11), and CMRL 1066 (7),<sup>3</sup> but since they gave no better or not as good

<sup>1</sup> CEE from 3 sources was used: (a) Difco Laboratories, Detroit 1, Mich. Control No. 437632; (b) Microbiological Associates, Inc., Bethesda, Md.; (c) several samples prepared in this laboratory by extraction of 8-10-day-old embryos with Hanks' balanced salt solution.

<sup>2</sup> Difco Laboratories, Detroit 1, Mich.

<sup>3</sup> Connaught Medical Research Laboratories, University of Toronto, Toronto, Canada.

results as No. 858 mdf, as prepared in this laboratory, their use was not continued.

In preparation of solution No. 1 of medium No. 858, cysteine HCl, glutathione, ascorbic acid, and glutamine were withheld and added directly to the completed medium at the time of mixing the several solutions. Serum was added usually in the proportion of 1 volume to 9 volumes of medium No. 858 mdf. Sufficient amounts were prepared at a time to last 3 or 4 days.

Calf serum was prepared in the laboratory from pooled samples of blood obtained from a local slaughter house and was sterilized by filtration through a Selas filter #02. Horse serum was obtained commercially.<sup>2</sup> Sera were stored at 9° C.

by an aliquot of the cell suspension. For culturing directly from the mouse the 60-mm. plates were used, and  $1 \times 10^6$  cells were added to each plate. The 30-mm. plates were used for subculture studies, and the number of cells used varied as desired. The cultures were incubated at 37° C. in a moist atmosphere which was continuously changed with a mixture of carbon dioxide and air in the proportions that would maintain the pH of uninoculated medium at about 7.2.

Cells for subcultures were obtained by rubbing cells loose from the bottom of the culture vessels with a wedge-shaped piece of silicone stopper affixed to the end of a dissecting needle.

*Cultures from single cells.*—Culture vessels 30

TABLE 1  
MODIFICATIONS OF MEDIUM NO. 858 (PER LITER)

Solution no.	Omissions	Additions
1	15 mg. Aspartic acid 100 mg. Dihydrostreptomycin SO <sub>4</sub> 1 mg. Penicillin G 10 mg. Adenine deoxyriboside 10 mg. Guanine deoxyriboside 10 mg. Cytosine deoxyriboside 10 mg. Thymidine	15 mg. Asparagine 25 mg. Streptomycin SO <sub>4</sub> 25 mg. Penicillin G 50 mg. Sodium deoxyribonucleate
2		10 mg. Niacin 10 mg. Riboflavin 10 mg. Thiamine HCl
4	21 mg. N-butylparahydroxybenzoate	
6	All ingredients	100 mg. Armour's liver coenzyme concentrate
7	10 mg. 5 Methyldeoxycytidine	

*Preparation of cells for culture.*—Cell-containing fluid, withdrawn from mice inoculated 5–7 days earlier, was diluted in a test tube with 4 or 5 volumes of either Earle's balanced salt solution (BSS) or the N-15 solution of Puck and co-workers (8). The cells were then sedimented at approximately  $300 \times g$  for 2 minutes in a small centrifuge. The supernatant fluid was discarded, and the cells were dispersed in the diluting fluid. Cell counts were made, and the suspension was diluted so that 0.1 ml. contained  $1 \times 10^6$  cells.

*Preparation of cultures.*—The complete medium, usually containing 10 per cent serum, was adjusted to pH 7–7.2 by placing the container (with a cotton stopper) in a closed vessel with a small piece of solid carbon dioxide until the pH was lowered as desired.

Four ml. of medium were placed in the 60-mm. plates and 2.0 ml. in the 30-mm. plates, followed

mm. in diameter were used for this purpose. An area about 20 mm. square on the outside of the bottom of a culture vessel was cross-hatched forming 36 squares about 3 mm.  $\times$  3 mm. each. For this purpose Marktex ink<sup>4</sup> was diluted with Xylol and used in a ruling pen. This ink dries quickly, adheres well, and is not damaged by alcohol and heat sterilization of the plates.

Cell-containing medium from cultures was diluted with fresh medium so that a small wire loop of the suspension contained about 25–75 cells as predetermined by microscopic examination of several droplets. One loop full of the suspension was dispersed in each plate containing 1.2 ml. of medium. The culture plates were then incubated for about 24 hours, at which time the medium was removed, the plate inverted and examined under low power of the microscope. The location of each

<sup>4</sup> Will Corporation, Rochester 3, N.Y.

cell adhering to the bottom of the plate was charted on paper. Usually there were some single cells adherent to the glass within some of the squares. More frequently twin cells, and sometimes more in groups, were attached to the bottom. After charting, the medium which had been removed was centrifuged to sediment any free cells and was then returned to the culture plate which was reincubated for another 24–36 hours. A reexamination under the microscope revealed whether any of the single cells had divided; if so the plates were observed daily until a selection of the best appearing colony could be made and then all other cells on the plate were rubbed loose. The loosened cells were then removed by rinsing the plate with cell-free medium. New medium was

Ehrlich ascites tumor were established from the same 74-day-old primary culture. The cells of the clones initially were uniform in appearance (Figs. 1–3), but as the culture aged considerable variability appeared among the cells.

The cells of several cultures were injected into mice in order to determine if they retained their malignant properties. All the Krebs cultures tested grew rapidly and caused death in about the usual period of time required by uncultured cells. One culture of the Krebs tumor from a single cell (117S3) isolated 40 days after initiation of the primary culture and maintained for an additional 115 days increased in mice 214-fold in 8 days. Of the two single-cell cultures of the Ehrlich ascites tumor  $3.25 \times 10^6$  cells of one were injected into each

TABLE 2  
GROWTH OF KREBS ASCITES CARCINOMA CELLS IN DIFFERENT MEDIA

MEDIUM NO.	COMPOSITION OF MEDIA				RESULTS
	Medium 858 mdf (per cent)	Calf serum (per cent)	Earle's BSS (per cent)	CEE-50-50 (per cent)	
1	0	40	40	20	All cells dead in 24–48 hours
2	84	10	0	6	All cells dead in 24–48 hours
3	84	10	6	0	Rapid growth
4	90	10	0	0	Rapid growth
5	100	0	0	0	No growth

The total volume of medium per culture was 4 ml., and the inoculum was  $1 \times 10^6$  cells.

then added to the plate and the colony allowed to proliferate.

### RESULTS

In a culture medium of calf serum, CEE and Earle's BSS (Table 2, medium #1) the Krebs ascites tumor cells failed to survive longer than 24–48 hours. Likewise in a mixture of medium No. 858 mdf, calf serum, and CEE (Table 2, medium #2) the cells died in 24–48 hours. When the CEE was omitted from the latter medium (Table 2, medium #3) excellent cultures were obtained. Medium No. 858 mdf without serum failed to support growth (Table 2, medium #5). Cultures of the Krebs ascites cells from fifteen different mice were initiated in medium #4, Table 2. One attempt was unsuccessful. The first of these cultures was still thriving after 8 months. Two attempts, both successful, were made to culture the Ehrlich (Lettré) ascites carcinoma cells in this medium.

Single cell cultures of the Krebs ascites tumor were established from original cultures 109, 96, 57, 40, and 30 days after preparation of the original cultures from the mice. Two such cultures of the

of two mice. In 8 days there was a 182-fold increase in the number of cells.

Primary cultures from different mice varied. Usually in 24–48 hours after the cells were added to the culture medium most of them had become adherent to the bottom of the plate, so that the medium could be removed. The plate could then be inverted and the cells observed under the microscope. Usually a variable small percentage of the cells had become enlarged and "vacuolated." Those eventually died and disintegrated. Most of the remainder of the cells appeared spherical, and some were extended on the glass surface.

The variation of the cultures from different mice in the initial stages was extreme on one occasion. Krebs ascites cells from two mice were plated, with the same sample of medium. One culture grew rapidly; there were few enlarged vacuolated cells, and the remaining cells were very uniform in appearance. That culture is thriving after 6 months. In the culture of cells from the other mouse about 10 per cent of the cells were of normal size and appearance; the remainder were extremely

enlarged and vacuolated. As the normal-appearing cells proliferated, others became enlarged and vacuolated and died. This condition persisted for 3 months when the culture was discontinued as a failure.

Apparently most of the cells became attached to the glass surface before undergoing mitosis. Cultures on coverglasses placed on the bottom of the culture vessels showed numerous mitotic figures (Figs. 3 and 4). Although mitotic cells were present in the supernatant fluid, it is uncertain whether or not such cells were free in the fluid when the mitotic process began. Growth of cultures in roller tubes at 50 r.p.h. was slow, and most of the cells were attached to the glass surface of the tubes.

Growth in the plate cultures was rapid. For example the medium (4 ml.) of a 60-mm. plate culture was renewed 52 times in 120 days, and an average of  $2 \times 10^6$  Krebs ascites cells was discarded each time in the supernatant fluid. In another experiment 400,000 cells were added to each of two 60-mm. plates, each containing 4 ml. of medium. At the end of 2 days the cells in the supernatant fluid of the two cultures were used to inoculate a third plate. At the end of 4 days the cells of the supernatant fluid of the three cultures were used for two additional cultures. At the end of 6 days the initial 800,000 cells had increased to  $26 \times 10^6$  cells, a 32-fold increase. In spite of its relatively strong buffering capacity the culture medium becomes rather acid in less than 48 hours. It is highly probable that more frequent, or continuous, renewal of the medium would induce a much higher growth rate.

Usually a small percentage of the cells in cultures was enlarged and vacuolated. Any adverse cultural conditions increased the percentage of those cells (Fig. 5). Correction of the condition resulted in a decrease (Fig. 6). As the population density increased the pH of the medium decreased, and the number of enlarged vacuolated cells increased. A large increase in the proportion of such cells was also noted during the last 2 of 10 weeks that a sample of calf serum was used (Fig. 5). A comparative test of that serum on small numbers of cells, with a sample of serum that had been stored frozen, showed that the serum in question would no longer fulfill the requirements for growth of small numbers of cells, whereas the frozen stored sample did. The use of a fresh sample of serum resulted in a prompt decrease in the numbers of enlarged, vacuolated cells (Fig. 6).

The results with horse serum<sup>5</sup> as a supplement to the modified 858 medium were inconsistent. On

some occasions cell growth was as good as that obtained with calf serum in the medium; on other occasions the medium was toxic and the cells were dead in 24 hours. The toxicity appeared to result from a reaction between some constituents of the horse serum and of the No. 858 mdf medium. When horse serum was added to freshly prepared No. 858 mdf medium and the medium used immediately, the medium was toxic. However, if the No. 858 mdf medium was prepared 24 hours or more before adding the serum and using the mixture, there was no toxicity.

#### DISCUSSION

The results of these studies show that chicken embryo extract is toxic in cultures of the cells of the Krebs ascites carcinoma and of the Ehrlich (Lettré) ascites carcinoma of mice and should not be a constituent of the culture medium. It may be noted that Graff and McCarty, who had the best results in culturing the Ehrlich ascites cells, did not use CEE in their culture medium. Calf serum supplements well the synthetic medium No. 858 modified, since small numbers of cells and even many single cell cultures of the Krebs tumor grew in the mixture. Horse serum was also satisfactory as a supplement if the precaution was taken not to add the serum to freshly prepared medium No. 858 mdf and then using the mixture immediately.

The presence of large vacuolated cells in the early stages of the growth of some primary cultures appears to indicate that the medium is not optimum for those cells. Either an adaptation of the cells to the cultural conditions or a selection of the fittest by elimination of those cells incapable of growth in the medium occurs, since the number of enlarged vacuolated cells usually decreases greatly as the time in culture lengthens provided the quality of the medium is maintained.

In order to initiate cultures from single cells it is essential that the serum used as a supplement be of good quality. Frequent testing of the serum as a supplement with very small numbers of cells is advisable.

#### REFERENCES

1. BARKA, T.; TARO, I.; POSALAKY, Z.; and PAPPAY, G. An Attempt To Grow Ehrlich's Ascites Tumor *in vitro*. *Acta Morphol. Acad. Sc. Hung.*, **6**:87-98, 1955-56.
2. EVANS, V. J.; BRYANT, J. C.; FIORAMONTI, M. C.; MCQUILKIN, W. T.; SANFORD, K. K.; and EARLE, W. R. Studies of Nutrient Media for Tissue Cells *in vitro*. *Cancer Research*, **16**:77-86, 1956.
3. EVANS, V. J.; BRYANT, J. C.; MCQUILKIN, W. T.; FIORA-

<sup>5</sup> Several samples from Microbiological Associates, Inc., Bethesda, Md., and one sample (Control No. 440784) from Difco Laboratories, Detroit 1, Mich.

- MONTI, M. C.; SANFORD, K. K.; WESTFALL, B. B.; and EARLE, W. R. Studies of Nutrient Media for Tissue Cells *in Vitro*. II. An Improved Protein-free Chemically Defined Medium for Long-term Cultivation of Strain L-929 Cells. *Cancer Research*, **16**:87-94, 1956.
4. GRAFF, S., and McCARTY, K. S. Sustained Cell Culture. *Exper. Cell Research*, **13**:348-57, 1957.
  5. HEALY, G. M.; FISHER, D. C.; and PARKER, R. C. Nutrition of Animal Cells in Tissue Culture. X. Synthetic Medium No. 858. *Proc. Soc. Exper. Biol. & Med.*, **89**:71-77, 1955.
  6. HULL, R. N. Establishing Long-term Cultures of Mammalian Normal, Solid Tumor, and Ascites Tumor Cells on Glass. *Science*, **117**:223-25, 1953.
  7. PARKER, R. C.; CASTOR, L. N.; and McCULLOCH, E. A. Altered Cell Strains in Continuous Culture: A General Survey. *In*: Conference on Cellular Biology, Nucleic Acids and Viruses. N. Y. Acad. Sc.: Spec. Publ. No. 5, pp. 303-13, 1957.
  8. POWELL, A. K. The Cultivation *in vitro* of Mouse Ascites Tumor Cells. The Relation between Density of Cell Population, Viability and Mitosis in "Pure Cultures." *Brit. J. Cancer*, **11**:274-79, 1957.
  9. PUCK, T. T.; CIECIURA, S. J.; and ROBINSON, A. Genetics of Somatic Mammalian Cells. III. Long-term Cultivation of Euploid Cells from Human and Animal Subjects. *J. Exper. Med.*, **108**:945-55, 1958.
  10. SIEGEL, B. V. *In Vitro* Cultivation of Mouse Ascites Tumor Cells for Virus-Host Cell Studies. *Nature*, **173**:584, 1954.
  11. WEYMOUTH, C. Rapid Proliferation of Sublines of NCTC Clone 929 (Strain L) Mouse Cells in a Simple Chemically Defined Medium (MB 752/1). *J. Nat. Cancer Inst.*, **22**:1003-17, 1959.

FIG. 1.—Krebs ascites carcinoma cells. Portion of a clone of single cell culture 117S1.  $\times 460$ .

FIG. 2.—Krebs ascites carcinoma cells. Portion of a clone of single cell culture 85S1.  $\times 460$ .

FIG. 3.—48-hour subculture of 117S1 in medium No. 858 mdf and 10 per cent calf serum. During the last 16 hours the cells were in contact with 2  $\mu$ g. per cent concentration of colchicine. Treated with hypotonic salt solution and stained with aceto-orcein.  $\times 460$ .

FIG. 4.—Krebs ascites carcinoma cells in culture 4 days from the mouse. The culture was treated as was that of Figure 3.  $\times 460$ .

FIG. 5.—Krebs ascites carcinoma cells showing enlarged, vacuolated cells, the result of using an old sample of calf serum.  $\times 460$ .

FIG. 6.—Same culture as shown in Figure 5, 8 days later using a new sample of serum in the medium.  $\times 460$ .

