Establishment of the TA₃ and Ehrlich Ascites Tumors as Permanent Cell Strains in Tissue Culture*

Muriel M. Guerin and Joseph F. Morgan

(Laboratory of Hygiene, Department of National Health and Welfare, Ottawa, Canada)

SUMMARY

Two mouse ascites tumors, the Ehrlich carcinoma and the TA₃ mammary carcinoma, have been established as permanent cell strains in tissue culture. Both cell strains have been carried by serial subcultivation through more than 60 passages during a 15-month period. The cell lines have been established and propagated in synthetic medium M 150, supplemented with a low level of calf serum. When inoculated into mice after prolonged tissue culture passage, both tumor cell strains elicited ascites tumor formation within 5–8 days in 90–100 per cent of the test mice.

Despite the widespread use of ascites tumors as test material for quantitative studies on the growth and biochemistry of neoplastic cells (11, 12), these tumors have not proved readily susceptible to establishment as cell strains in tissue culture. In 1953, Hull (9) described the serial cultivation of Ehrlich ascites cells on glass for a limited period of time, and Gey (6) reported some success in propagating Ehrlich cells, following intraperitoneal passage through rats. Powell (17, 18) observed that a protective effect exerted by explants of various animal tissues or by normal spleen monocytes was essential to promote the growth in vitro of Ehrlich and Sarcoma 37 ascites tumor cells. DeSchner and Allen (1) studied the Ehrlich tumor in tissue culture but were unable to demonstrate tumor formation when their cell line was inoculated into mice. Jackson, Giuffre, and Perlman (10) and Foley and associates (5) have recently reported establishment of the Ehrlich ascites tumor in tissue culture, but, again, attempts to cause tumor formation in mice following inoculation of the cell line were not successful (10). Other ascites tumors recently established as cell strains have been the 6C3HED mouse lymphosarcoma by Guerin and Kitchen (7) and the Yoshida and AH-130 rat ascites hepatoma by Takaoka and Katsuta (19).

Studies have been in progress in this laboratory* to investigate the antitumor activity of fatty acids (16, 20, 21), with ascites tumors in mice used as the test system. It was deemed of value to extend this study to include the effect of fatty acids on malignant cell cultures, and, to this end, attempts were made to establish the ascites cells in tissue culture. The present communication reports the serial propagation in vitro of the TA₃ mammary carcinoma and the Ehrlich carcinoma, and their establishment as permanent cell strains. The formation of ascites tumors in mice following inoculation of these established cell strains is also described.

MATERIALS AND METHODS

Tumors.—Stocks of the TA₃ and Ehrlich ascites tumors were maintained in storage at -70°C following rapid freezing in 20 per cent glycerol by the procedure of Morgan, Guerin, and Morton (14). After thawing, both tumors were carried by weekly serial passage in the Connaught mouse line of A strain origin (14). During the routine passages each mouse received an intraperitoneal inoculation of 20–25 million washed tumor cells. The TA₃ tumor had been stored for 23 months prior to revival and was passaged serially in mice for 5 months before the start of the experiments. Freshly harvested ascitic fluid of the twentieth mouse passage of this series was used as the source of TA₃ cells for the cultivation attempts. The Ehrlich tumor had been stored for 6 months and was passaged serially for 6 months prior to these experiments. Ascitic fluid from the 29th mouse
passage of this series supplied the Ehrlich cells for cultivation.

Culture media and materials.—Synthetic tissue culture medium M 150 (13) (a modification of medium 199 [15]), supplemented with 5–20 per cent pooled calf serum, was used throughout these experiments. Hanks balanced salt solution (8) was used as the inorganic base of the medium. Calf blood was obtained from a local abattoir, allowed to clot in the cold, the serum removed, filtered through UF fritted glass filters, and stored at –20°C until required. Trypsin (1:250) was obtained from Difco Laboratories, Inc., Detroit, Michigan, and versene (ethylenediamine tetra-acetate, disodium) from the Bersworth Chemical Co., Framingham, Massachusetts. Culture vessels employed were flasks of the T series, as described by Earle and Highhouse (2). The mice used in these experiments were purchased from the Connaught Medical Research Laboratories, University of Toronto.

Primary cultures.—Ascitic fluids were withdrawn aseptically from mice 5–8 days following routine tumor inoculation. The fluids were mixed with 2 volumes of sterile distilled water to lyse any erythrocytes and centrifuged at 2000 r.p.m. for 3 minutes in graduated conical centrifuge tubes. The supernatant fluid was discarded. The sedimented cells were resuspended in approximately 4 volumes of medium M 150 and centrifuged again at 800 r.p.m. for 3 minutes. The packed cells were resuspended in a small volume of medium M 150, counted in a hemocytometer, and aliquots containing approximately 6 × 10^6 cells distributed into T 60 flasks. Half this number of cells was seeded into T 30 flasks.

Culture medium M 150, supplemented with 10 per cent calf serum, was added to each culture (10 ml per T 60 and 5 ml per T 30 flask), and the flasks were incubated at 37°C in a stationary position. After 24 hours the culture medium was removed completely and replaced with medium M 150 containing 10 per cent calf serum. All cultures were examined daily under the microscope, and fluid changes were made at 1–2-day intervals, depending upon the pH of the medium.

Subcultures.—In the early tissue culture passages, cells were removed from the glass surfaces by replacing the culture fluids with an equal volume of Hanks balanced salt solution (8) containing 0.25 per cent trypsin and shaking the flasks gently at room temperature. During later passages, a 0.02 per cent versene solution (7) was substituted for the trypsin without deleterious effects.

RESULTS

Establishment of cultures.—Six to 7 days after the initial seeding of the flasks with ascites tumor cells, subcultivation attempts were begun. Chart 1 indicates diagrammatically the passage procedure followed during the establishment of the TAA strain. Cells from the two original T 60 flasks were removed with trypsin, centrifuged, resuspended in fresh medium M 150 containing 10 per cent calf serum, and dispensed into a single T 30 flask. This reduction of the growth surface area was made to maintain the inoculum of viable cells at a high level, as recommended by Earle and associates (3). After 21 days, a second “reduced subculture” was made to a single T 15 flask. At this point, the TAA cells began to show definite proliferation on the glass surface, and within 10 days it became possible, on subculture, to increase the line to two T 15 flasks. Following this passage, successive transfers were carried out on progressively increasing surface areas, with the use of larger flasks or increased numbers of small ones. After the sixth such transfer of the TAA cells, subcultures could be made regularly at 5- to 8-day intervals, and the cell line was considered established.

The step-by-step procedure in the establish-
ment of the Ehrlich cell line is illustrated in Chart 2. As in the case of the TA3 cells, a reduction in glass surface area on subculture was found necessary. It is evident (Chart 2) that the Ehrlich cells were much slower than the TA3 cells in evincing active proliferation. One attempt to increase the culture surface after the third transfer was unsuccessful. Only a few viable cells could be found in the one remaining T 15 flask, and this culture was held for 51 days before transfer to a fresh T 15 flask was made. After this transfer, the cell population remained low for approximately 21 days and then increased slowly. By 32 days, a definite increase in cell population was evident, and the culture area was increased by transfer to two T 15 flasks. Following this passage, the cell population increased rapidly, and the cultures could be transferred routinely at 6- to 8-day intervals. At this point, the strain was regarded as established.

Both tumor cell lines have now been in continuous cultivation for more than 15 months. The TA3 line has undergone more than 70 serial passages and the Ehrlich line more than 60 passages. The morphological appearance of these two strains immediately after subcultivation and after 5 days' propagation is shown in Figures 1-4. The Ehrlich strain (Figs. 1 and 3) is characterized by large, fibroblast-like spindle cells, whereas the TA3 strain (Figs. 2 and 4) consists of smaller cells with a mixture of rounded and triangular forms.

**Tumor formation by ascites cell cultures.**—To determine whether serial cultivation *in vitro* had resulted in loss of tumor-inducing capacity, both cell lines were inoculated intraperitoneally into mice. Cells from the tenth tissue culture passage of the Ehrlich line and from the sixth and twentieth tissue culture passages of the TA3 line were selected for transplantation studies. These passages were selected to represent early, intermediate, and prolonged periods of cultivation *in vitro*. The cells were harvested from culture flasks, resuspended in a small volume of medium M 150, counted, and aliquots containing 2 to 3 X 10^6 cells were injected intraperitoneally into groups of Connaught mice. A tumor incidence of 90-100 per cent was obtained in each series of inoculations, and the resulting tumors were carried through five to seven serial mouse passages. In all cases, typical ascites formation was obtained in 6-8 days.

**DISCUSSION**

The present experiments have demonstrated the establishment, as continuous cultures *in vitro*, of the TA3 and Ehrlich ascites tumor cells. This appears to represent the first cultivation of the TA3 tumor cell *in vitro* and the first unequivocal long-term propagation of the Ehrlich ascites tumor in tissue culture. In view of the difficulties reported by other workers with this tumor cell (1, 17, 18), it should be noted that both the Ehrlich and TA3 tumors had been subjected to the freezing procedure of Morgan, Guerin, and Morton (14) prior to these experiments. This method was also employed by Guerin and Kitchen (7) in the successful establishment of the 6C3HED mouse lymphosarcoma cell in tissue culture. Since this freezing procedure alters the mouse-strain specificity of the ascites cells (14), it may conceivably contribute to the ease of their subsequent establishment in tissue culture. Experiments to test this possibility are now being carried out with ascites cells having no history of freezing treatment.

Establishment of both the TA3 and Ehrlich cell lines in this laboratory was accomplished in synthetic medium M 150, supplemented with varying amounts of calf serum. Once the cell strains had become stabilized, it was possible to reduce the serum content of the medium to a very low level (2 per cent) with no apparent reduction in the propagation rate. In the absence of serum, the cell lines could be maintained in completely synthetic medium for at least 5-6 days before cell degeneration became evident, a period suf-
ficiently long to permit determination of specific nutritional requirements. Initial studies (to be published) have indicated that even minor alterations in the organic components of synthetic medium M 150 have strongly adverse effects on both cell lines. These findings are in marked contrast to the observations of Eaton and Scala (4), who reported only limited survival of Ehrlich cell cultures in synthetic medium 199 and did not obtain proliferation of the cultures upon supplementation with serum protein. Since the difference between medium M 150 (13) and medium 199 (15) lies only in the basic salt solution, it would appear that variations in technique are also critical with the ascites cell cultures.

An important step in the establishment of both the TA3 and Ehrlich ascites strains was the use of a "reduced subculture" method during the initial passages of the cells. By this procedure, the surface area of the cultures was reduced progressively, and the number of viable cells was kept high in proportion to the glass surface area. Once cell proliferation was initiated, normal passage to equal or larger surface areas became possible. This principle of "reduced subculture" was introduced originally by Earle and his associates (3) in studies with the L strain and does not appear to have been applied previously to cultivation of ascites cells.

It is of interest to note that inoculation of mice with cells derived from cultures after six, ten, and twenty subcultivations in vitro produced ascites tumors within the normal time period and that the resulting tumors could be passaged serially in mice. It is evident, therefore, that establishment of these cells in tissue culture has not resulted in the ascites cell cultures.

The establishment of these two tumor strains as continuous cell cultures with unimpaired tumor-inducing capacity now makes it possible to study the antitumor activity of various compounds on the same neoplastic cell under comparable in vitro and in vivo conditions.

ACKNOWLEDGMENTS

We wish to acknowledge our indebtedness to Mrs. J. Clark and Mrs. M. Thomas, whose technical assistance helped materially in the completion of these experiments.


Figs. 1-4.—General morphological characteristics of established Ehrlich and TA\textsubscript{3} ascites cell lines. All photographs taken at 240X magnification.

Fig. 1.—Ehrlich strain in 38th tissue culture passage, photographed 24 hours after transfer.

Fig. 2.—TA\textsubscript{3} strain in 57th tissue culture passage, photographed 24 hours after transfer.

Fig. 3.—Ehrlich culture 1, photographed 5 days after transfer.

Fig. 4.—TA\textsubscript{3} culture 2, photographed 5 days after transfer.


FIG. 1.--General morphological characteristics of established Ehrlich and TA3 ascites cell lines. All photographs taken at 40X magnification.

FIG. 1.--Ehrlich strain in 38th tissue culture passage, photographed 24 hours after transfer.

FIG. 2.--TA3 strain in 57th tissue culture passage, photographed 24 hours after transfer.

FIG. 3.--Ehrlich culture 1, photographed 5 days after transfer.

FIG. 4.--TA3 culture 1, photographed 5 days after transfer.
Establishment of the TA3 and Ehrlich Ascites Tumors as Permanent Cell Strains in Tissue Culture

Muriel M. Guerin and Joseph F. Morgan