Differentiation of "Normal" and Neoplastic Cells Maintained in Tissue Culture by Implantation into Normal Hamsters.* (23044)

G. E. FOLEY AND A. H. HANDLER (Introduced by Sidney Farber)
Laboratories of Microbiology and Tumor Transplantation, Children's Cancer Research Foundation, and Department of Pathology, Harvard Medical School, at Children's Medical Center, Boston, Mass.

It is generally accepted that cell lines derived from normal tissue and those derived from neoplastic tissue are difficult to distinguish from one another after maintenance in tissue culture(1,2). Experiments were therefore designed to ascertain if biological differences between such cell lines could be demonstrated by application of newer methods of tissue culture(3-8) and transplantation of tissue culture cell lines to a heterologous host(9). Delineation of specific amino acid and vitamin requirements of a mouse fibroblast(3) and a human carcinoma cell line(4) has permitted parallel *in vitro* cultivation of several additional human and animal cell lines deriving from normal and neoplastic tissue(5-8) in media embodying these same minimal requirements supplemented with 10% whole serum. Studies with these cell lines *in vitro* have failed thus far to reveal significant biochemical or morphological differences between those derived from normal and from neoplastic tissue. Similarly, studies on susceptibility of cell lines originating from normal *vs.* malignant tissue to certain carcinolytic agents(10) resulted in the paradoxical observation that either was equally susceptible to a given agent, despite the fact that these same compounds exhibit selective anti-tumor activity in experimental animals. Observations during the past 18 months on quantitative titration of tissue culture cell lines in the cheek pouches of golden hamsters(9) suggested that all cell lines do not behave similarly in this heterologous host. The purpose of this report is to record the results of experiments which indicate that despite the aforementioned *in vitro* similarities, there are significant differences in the biological behavior of different cell lines maintained *in vitro*, and that these differences may correlate with the source of the culture.

Methods. The cell lines studied are listed in Table I. These strains were maintained in media prepared as described by Eagle(3-5) in which the essential serum protein was provided by 10% whole, pooled, human serum, except for 4 animal cell lines, where serum protein was provided by 10% whole horse serum. All of these cell lines exhibit remarkably similar rates of growth in these media, and with the exception of Sarcoma 180 and L-929, which are of fibroblast-like morphology, resemble one another in morphologic appearance. They are best described as "epithelial-like" in the sense that individual cells are predominantly polygonal, with distinct, intensely basophilic nuclei, and their characteristic growth in essentially monocellular layers directly on glass surfaces gives the appearance of "pavement" epithelium. The layer of cells growing on the surface of culture flasks was removed for animal experiments by replacing the culture fluid in 5-7-day-old cultures with fresh media containing Difco "1:250" trypsin in final concentration of 0.125% and incubating the flask *circa* 5 min at 37°C. The cells so dispersed were sedimented at 5-600 rpm, washed 2-3 times with fresh medium to remove the trypsin and redispersed with gentle agitation in appropriate volumes of media. After enumeration by haemocytometer counts, the suspension of cells was diluted so that the desired inoculum was contained in a volume of 0.1 ml of medium. Syrian hamsters, 60-80 g in wt were prepared under light nembutal anaesthesia and the desired inoculum implanted under the epithelium of the cheek pouch with a 24-gauge needle. Both cheek pouches of 6 hamsters were implanted with each inoculum of each cell line and in each instance, 3 of 6 hamsters were treated with

* This investigation was supported in part by research grants from Natl. Cancer Inst., N.I.H., U. S. Public Health Service.

TABLE I. Titration of Tissue Culture Inocula in Unconditioned Hamsters.

Cell line	Tissue of origin	*In vitro* age (mo)†	Development of tumors* following implantation of cells at: 1×10^6	1×10^5	1×10^4
Sarcoma 180	Biopsy, S-180 in CFW mice (Foley)	18	+	+	+
KB‡	Biopsy, human, epidermoid carcinoma (Eagle)	23	+	+	+
HeLa‡	" " cervical " (Gey)	69.5	+	+	+
J-111‡	Peripheral blood, human, acute monocytic leukemia (Osgood)	27	+	+	+
Wilms'-6	Biopsy, human, Wilms' tumor (Foley)	4.5	+	+	+
Amnion	Human amnion at term (Foley)	3.5	+	+	0
407‡	" embryonic intestine (Henle)	23	+	+	0
LLC-M-1§	Hypertrophic lymph node, mouse of unknown strain (Hull)	61	+	0	0
L‡	Biopsy, human, liver (Chang)	31	+	0	0
Thymus	" " thymus (Foley)	5.5	+	0	0
Endometrium	Normal, human, therapeutic abortion (Foley)	5.5	+	0	0
Lung	Biopsy, human, lung‖ (Foley)	5.5	+	0	0
L-929	Connective tissue, C3H mouse (Earle)	194	+	0	0
Kidney¶	Normal Rhesus monkey	.5	+	0	0

* See text.
† To Dec. 1, 1956.
‡ Strains obtained through courtesy of Dr. Harry Eagle, N.I.H., Bethesda, Md.
§ *Idem*, Dr. R. N. Hull, Eli Lilly Research Labs., Indianapolis, Ind.
‖ Chronic pneumonitis.
¶ Primary *in vitro* cultures. Trypsin-dispersed suspensions of 1×10^5 and 1×10^4 cells implanted directly also failed to grow in unconditioned hamsters.

cortisone acetate in subcutaneous doses of 2-3 mg, administered simultaneously at time of implantation and twice weekly post-implantation. *The 3 remaining hamsters in each instance were not treated with cortisone.* At least 2 such experiments were done with each cell line, thus the data summarized in Tables I and II represent observations on *circa* 800 hamsters. Cheek pouches of all animals were observed twice-weekly under light nembutal anaesthesia for 60 days. Development of a nodule which increased progressively in size and became vascularized within a few days was considered as evidence of growth. All cell lines recorded as positive in Table I produced such tumors in two or more cheek pouches per experiment in more than one animal. These tumors may regress spontaneously in unconditioned hamsters, but are readily transplanted to other hamsters within the first 10-14 days. Histological sections were prepared from all cheek pouches in which such tumors developed. The behavior of cell lines recorded as negative in Table I was distinctly different. In most instances, the original implant disappeared completely within 24 hrs, but occasionally persisted as a tiny nodule which regressed spontaneously within 5-6 days. In no instance did the original implant develop progressively or become vascularized, and they were not transplantable to other hamsters. Histologically, such nodules consist of a necrotic focus at site of implantation, surrounded by the usual inflammatory response.

TABLE II. Titration of Tissue Culture Inocula in Hamsters Conditioned with Cortisone Acetate.

Cell line†	Development of tumors* following implantation of cells at: 1×10^5	1×10^4	1×10^3	1×10^2	10
S-180	+	+	+	+	+
KB	+	+	+	+	+
HeLa	+	+	+	+	+
407	+	+	+	0	0
L	+	+	0	0	0

* See text. † See Table I.

Results. All cell lines studied, irrespective of tissue of origin, grew prolifically in cheek pouches of hamsters conditioned with cortisone acetate when the inoculum contained 1 x 10^5 or more cells. However, *as summarized in Table I, this was not the case with unconditioned hamsters.* Although each cell line so studied grew when 1 x 10^6 cells were implanted in cheek pouches of unconditioned

hamsters, results with inocula containing 1 x 10^5 and 1 x 10^4 cells clearly indicate that all strains which can be maintained *in vitro* do not exhibit identical *in vivo* biological properties. Thus far, the failure of 1 x 10^5 cells in most instances, and the uniform failure of 1 x 10^4 cells to grow in unconditioned hamsters appears to be characteristic of those cell lines deriving from non-malignant sources. That this failure to grow in unconditioned hamsters is not entirely due to length of time the cell line has been in tissue culture is evident from Table I, since long-established (LLC-M-1, L, L-929) as well as more recently isolated (Thymus, Endometrium, Lung) cell lines failed to grow under these experimental conditions.

In sharp contrast, all cell lines deriving from neoplastic tissue produced characteristic tumors in 33-100% of cheek pouches of unconditioned hamsters implanted with 1 x 10^5 or 1 x 10^4 cells, and a number of tumors so induced have been maintained through several transplants in unconditioned hamsters. The uniform ability of such cell lines to grow under these experimental conditions also is noteworthy, since it is conceivable that the tumor from which these cells were isolated may have contained normal stromal cells. Again, this ability to grow in unconditioned hamsters is not simply a reflection of length of time the cell line has been maintained *in vitro,* as evidenced by comparison of *in vitro* ages of strains KB, HeLa, and J-111 with those of S-180 and Wilms' 6.

Experiments in which tissue culture inocula were titrated further in cheek pouches of hamsters conditioned with cortisone acetate give further evidence of differences in biological behavior of cell lines derived from different sources. Thus far (Table II) cell lines isolated from neoplastic tissue produce tumors following implantation of as few as *circa* 10 cells, whereas cell lines derived from adult non-malignant tissue fail to produce tumors, even in conditioned hamsters, when inocula of 1 x 10^3 or fewer cells are used. The strain of normal embryonic cells so studied (407, Table II) exhibits a growth potential intermediate between that of cell lines derived from malignant and adult non-malignant tissue. Further studies on the titration of these and additional cell lines, including a number isolated from aspirated bone marrow of patients with and without neoplasm(6), in conditioned and unconditioned hamsters are in progress.

Discussion. The factors of adaptation and/or selection determining successful isolation of mammalian cells *in vitro* have not yet been fully delineated. Cell lines exhibiting different growth potentials have been isolated from cultures derived from malignant(11), and non-malignant sources(12-14). Thus, it is of considerable interest that morphologically similar tissue culture cell lines exhibit distinctly different biological properties when exposed to physiological influences of an intact heterologous host. Differences in behavior in unconditioned hamster cheek pouches of cell lines used in studies on cytotoxic activity of carcinolytic agents in tissue culture(10) suggest that despite the similarity in response to such agents *in vitro,* significant biological differences between these cell lines indeed exist, and that further *in vitro* study of such cell lines may reveal biochemical attributes related to these differences in growth potential.

It is perhaps more than coincidence that cell lines derived from neoplastic tissue exhibit a greater growth potential than those derived from non-malignant sources when titrated in unconditioned hamsters. Moore *et al.*(15) mentioned that cell lines derived from normal sources failed to grow in unconditioned rats, while previous experience in these laboratories with neoplastic or embryonic and heterologous adult normal tissue implanted directly into the hamster cheek pouch by trocar or as trypsin-dispersed cell suspensions indicates that under these conditions, adult normal tissue fails to grow. These observations are in accord with Greene's suggestion(16) of heterotransplantability as a criterion for distinguishing between neoplastic (or embryonic) and adult normal tissue. The present studies, however, must be extended to additional cell lines and the results correlated with behavior of tissue cells under other biological conditions both *in vivo* and *in vitro* before

differences in growth potential exhibited in the unconditioned hamster can be evaluated as differential evidence of malignancy in general. However, the present data can be interpreted as representing distinct biological differences among those cell lines derived from normal and neoplastic tissues which can be maintained in tissue culture.

Summary. Quantitated inocula of 14 tissue culture cell lines which are remarkably similar *in vitro* were implanted into cheek pouches of unconditioned Syrian hamsters. All cell lines grew in the cheek pouch when 1 x 10^6 cells were implanted, but only those cell lines derived from neoplastic tissue produced tumors when inocula contained 1 x 10^4 cells. Other experiments indicated that this difference in the growth potential of cell lines deriving from neoplastic tissue also may be delineated in hamsters conditioned with cortisone acetate by implantation with 1 x 10^3 or fewer cells.

1. Parker, R. C., *Methods of Tissue Culture,* 1950, Ed. 2, P. B. Hoeber, Inc., New York.

2. Leighton, J., Kline, I., Belkin, M., Legallais, F., and Orr, H. C., *Anat. Rec.,* 1956, v124, 492.

3. Eagle, H., *J. Biol. Chem.,* 1955, v214, 839.

4. ———, *J. Exp. Med.,* 1955, v102, 37.

5. ———, PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 362.

6. Herman, L., and Stulberg, C. S., *ibid.,* 1956, v92, 730.

7. Foley, G. E., and Drolet, B. P., *ibid.,* 1956, v92, 347.

8. Eagle, H., Oyama, V. I., Levy, M., and Freeman, A., *Science,* 1956, v123, 845.

9. Handler, A. H., and Foley, G. E., PROC. SOC. EXP. BIOL. AND MED., 1956, v91, 237.

10. Eagle, H., and Foley, G. E., *Am. J. Med.,* 1956, v21, 739.

11. Puck, T. H., Marcus, P. I., and Cieciura, S. J., *J. Exp. Med.,* 1956, v103, 273.

12. Leighton, J., Kline, I., and Orr, H. C., *Science,* 1956, v123, 502.

13. Earle, W. R., Nettleship, A., *et al., J. Nat. Cancer Inst.,* 1943, v4, 213.

14. Sanford, K. K., Likely, G. D., and Earle, W. R., *ibid.,* 1954, v15, 215.

15. Moore, A. E., Southam, C. M., and Sternberg, S. S., *Science,* 1956, v124, 127.

16. Greene, H. S. N., *J. Am. Med. Assn.,* 1948, v137, 1364.

Received December 27, 1956. P.S.E.B.M., 1957, v94.

Antagonism of Insulin-Induced Gastrointestinal Hypermotility in the Rat.* (23045)

PAUL M. LISH AND EMMETT L. PETERS (Introduced by M. R. Warren)
Department of Pharmacology, Mead Johnson Research Laboratories, Evansville, Ind.

Antispasmodic potencies of many drugs have been expressed in terms of relative degree of antipropulsive action shown in the Macht and Barba-Gose charcoal meal test(1). The results of this test are informative, but effects of drugs on normal propulsive motility may not reflect useful effects expected in treatment of gastrointestinal malfunctions associated with smooth muscle spasm and hypermotility. Some clinically useful agents, such as adiphenine hydrochloride (Trasentine) and dicyclomine hydrochloride (Bentyl), which exert their smooth muscle inhibiting effect in "musculotropic" or "papaverine-like" fashion, are without significant inhibitory effect in the charcoal meal test except in doses which produce pronounced central nervous system effects. Atropine sulfate and methantheline bromide (Banthine) require subcutaneous doses of 2 mg/kg and 15 mg/kg respectively to produce a 50% decrease in normal propulsion. Such high doses of these agents might be expected to be responsible for extraneous effects which could be antagonistic or synergistic with peripheral antipropulsive action of the compound. The charcoal meal test alone fails to indicate ex-

* We wish to express our sincere thanks to Dr. Marshall R. Warren and Dr. J. P. Quigley for advice concerning preparation of manuscript.