The Tumor-producing Capacity of Strain L Mouse Cells after 10 Years *in Vitro*

KATHERINE K. SANFORD, GWENDOLYN LIKELY HOBBS, AND WILTON R. EARLE

(Tissue Culture Section, Laboratory of Biology, National Cancer Institute, Bethesda, Maryland)*

It has been generally concluded that tumor cells grown in tissue culture indefinitely retain their ability to produce tumors when injected into animals of the strain from which the tissue originated. Thus, Lewis (15) found that six different rat sarcomas that had been grown in heterologous culture media for periods of 2 to over 4 years produced typical sarcomas when inoculated into rats from time to time during the period of cultivation. Fischer and Davidsohn (8) cultured a mouse adenocarcinoma for a period of 12 years in a heterologous culture medium, testing the cells periodically by mouse injection. They concluded that the malignant property of the cells remained almost constant during the period of cultivation; in spite of seasonal variations in takes, approximately 75 per cent of the mice inoculated developed tumors.

An apparent exception to the above conclusion was reported in 1950 by Earle, Shelton, and Schilling (5), who found that with increased time in tissue culture a progressive decrease occurred in the ability of six mouse cell strains to give rise to sarcomas when injected into animals of the inbred strain of origin. These six cell strains were originated in October 1940 (2) from an explant of subcutaneous connective tissue taken from a strain C3H (Andervont substrain) mouse. After 2 years of growth *in vitro*, cultures from all six strains were found to give rise to sarcomas when injected into strain C3H mice; during the subsequent 4 years of testing, however, all strains showed in general a progressive lowering of tumor-producing ability (5). The incidence of sarcomas produced by one of the strains, strain L, dropped from 68 per cent in 1943 to 1 per cent in 1946.

During this period, some experiments were carried out by Algire, Chalkley, and Earle (1) to determine what correlations might exist between the ability of the six cell strains to give rise to sarcomas and the vascular reaction of the host to implants of the cell strains; the implants were made into transparent chambers *in vivo*. In the course of these experiments a change was noted in one of the six tissue culture strains, strain L. In the initial experiments of 1943 and 1944, the strain L cell mass became well vascularized and remained translucent during the 2–3-week period of observation. Several years later, however, in 1948, when a few additional transplants were made into the same inbred strain of mice, vascularization and growth of the strain L cell mass for 8–10 days were followed by increasing opacity and regression of the graft. The reaction was similar to that encountered when tumor tissue is transplanted to the subcutaneous tissue of a resistant mouse strain. This finding suggested that an incompatibility had developed between the tissue culture cells and the host strain of mouse.

In 1948 a single cell was isolated from a culture of strain L; from this a line of cells designated clone 929 was obtained (18). This clone has been examined in the present experiments with the following objectives in mind:

1. To determine whether clone 929 cells are still capable of producing sarcomas in strain C3H mice;
2. To establish whether clone 929 cells induce an immune reaction in the strain C3H mouse;
3. To determine whether clone 929 cells can be adapted to grow progressively in 100 per cent of strain C3H mice inoculated; and
4. To test whether clone 929 cells can grow in other inbred strains of mice.

MATERIALS AND METHODS

Clone 929 cells were grown under sheets of perforated cellophane in T-60 flasks (3) according to culture methods previously described (6). The culture medium consisted of 40 per cent horse serum, 20 per cent chick embryo extract, and 40 per cent Earle's saline. Cultures grown for 10–14 days containing approximately 3×10^7 cells per culture were used for injection; each culture was usually injected into two mice. In preparation for injection, the cells were shaken from the surface of the cellophane and from the glass floor of the flask,

* National Institutes of Health, Public Health Service, U.S. Department of Health, Education and Welfare.

Received for publication September 23, 1955.

and the cell suspension in culture fluid was centrifuged at 33 × *g* for 10 minutes. After centrifugation the cell pellet was resuspended in the appropriate volume of supernatant culture fluid; 1 ml. of cell suspension was injected into each mouse.

The procedure for intramuscular injection of tissue culture cells and for subinoculation of tumors was similar to the technic described earlier (4, 5). In the subinoculation of tumors, the tumor was removed aseptically, and small fragments of tissue were implanted by trocar into the thigh muscles. The mice used were males usually of 1–2 months of age of strain C3H/HeN, originally Andervont's substrain.

Some mice were x-radiated 3–6 hours before injection. The irradiation was administered from a double tube as a single, whole-body dose, usually 425 r.[1] Physical factors were as follows: 186 kv. (peak), 170 v., and 20 ma., 96.7 r.p.m. at a focal distance of 54 cm. through 0.25-mm. Cu and 1.10-mm. Al filters.

In the immunization experiments, strain C3H/HeN mice, 1–2 months old, were immunized by injecting 1 ml. of a dense suspension of clone 929 cells in culture fluid into the muscles of the foreleg. One culture of cells was usually used for one to three mice. After 2 weeks the immunized mice as well as control nonimmunized mice of the same age were subjected to whole-body x-radiation; a single dose of 425 r was used. A few hours after x-radiation both control nonimmunized and immunized mice received an injection in the muscles of the left thigh of 1 ml. of a suspension of clone 929 cells. The suspension was prepared by pooling the cells of several cultures to yield approximately 1.5–3 × 10^7 cells/ml.

After injection, all mice were examined weekly for a period of 3–4 months. Tumors were allowed to reach at least 1 cm. in diameter and were usually considerably larger before autopsy of the mouse. Autopsies were performed on all mice except those that were occasionally found dead and were too badly decomposed to autopsy. Strips of tumor tissue were fixed in formalin-Zenker, sectioned, and stained with hematoxylin and eosin, and Van Gieson's picro-fuchsin. Identification of tumors was made by microscopic examination of the sections.

[1] The authors wish to express their appreciation to Mr. Henry L. Meyer of the Radiation Branch of this Institute, who irradiated the animals.

RESULTS

Experiments to test the tumor-producing capacity of clone 929 cells.—Since whole-body x-radiation is known to lower the resistance of the host to tumor implants (17), clone 929 cells were injected into both x-radiated and nonirradiated mice. The results of these injections are summarized in Table 1. Cells that had been cultured for periods ranging from ½ to 5 years after isolation of the original single cell of clone 929 were injected into 72 mice; 15 per cent (eleven mice) of the 71 that survived developed tumors at the injection site. These tumors were palpable 6–15 weeks after injection of the tissue cultures and were diagnosed as sarcomas.

Cells cultured for periods ranging from 4 to 5 years after single cell isolation were injected into 39 x-radiated mice. In the last three groups of injections into irradiated animals, one half of each culture was injected into an irradiated mouse and one half into a nonirradiated mouse. Of the 39 irradiated mice injected, 64 per cent (25 mice) developed tumors at the injection site. These were palpable 2½–12 weeks after injection of the tissue cultures. All these were diagnosed as sarcomas; thus the incidence of sarcomas produced by injecting clone 929 cells into nonirradiated animals was 15 per cent and into x-radiated mice was increased to 64 per cent.

Experiments to test whether clone 929 cells induce an immune reaction in the strain C3H mouse.—The results of these experiments are presented in Table 2. The data indicate that a prior injection of clone 929 cells increased the resistance of the strain C3H mouse to clone 929 cells to such an extent that no tumors developed in the immunized

TABLE 1

RESULTS OF INJECTING CULTURES OF CLONE 929 INTO X-RADIATED AND NONIRRADIATED STRAIN C3H MICE

Months in culture from single cell isolation	Transplant generation of cultures	No. cultures injected	Nonirradiated mice: No. injected	Nonirradiated mice: No. with sarcomas*	X-radiated mice: No. injected	X-radiated mice: Age (days)	X-radiated mice: Radiation dose (roentgens)	X-radiated mice: No. with sarcomas*
6⅔	10	3	3	0/3				
10⅓	16	3	11	1/11				
50	103	1			4	39	450	1/1
51	106	5			5	40	400†	4/4
54½	113	5	10	4/10	10	37	495	0/0
55	115	8	8	1/8	8	34	400	5/8
56⅔	118	9	9	1/9				
57	119	10	10	0/10	10	38	400	5/9
59½	124	11	11	3/11	11	32	350	4/9
60¾	127	10	10	1/9	10	35	400	6/8
Totals:		65	72	11/71 (15 per cent)	58			25/39 (64 per cent)

* The denominator represents the number of mice surviving and the numerator the number developing sarcomas.

† Received a second dose of 400 r 22 days later.

animals, whereas 56 per cent of the nonimmunized control animals developed sarcomas at the test site.

Experiment to test whether clone 929 cells can be adapted to grow in 100 per cent of strain C3H mice inoculated.—Sarcomas arising in x-radiated strain C3H mice and in one nonirradiated mouse were subinoculated to determine whether any tumor could be adapted to grow progressively in all strain C3H mice injected. Eighteen tumors were carried for one to eight generations *in vivo* in mice that had received a whole-body dose of 425 r before injection of the tumor tissue. Tumors arising in these mice were tested for their ability to grow in nonirradiated strain C3H mice.

The results of this experiment are summarized in Table 3. Most of the tumors used for subinoculation were taken from a stock of 158 x-radiated mice, 78 per cent of which developed sarcomas at the injection site. The remaining tumors were subinoculated from nonirradiated mice as indicated.

Of the eighteen sarcomas tested, two during the third generation *in vivo* were found to grow progressively in all mice injected. One of these that originated in a nonirradiated mouse was carried for an additional five generations *in vivo;* sarcomas developed in all mice injected.

Experiment to test whether clone 929 cells can grow in other strains of mice.—An effort was made to determine whether clone 929 tumor cells could grow progressively in other strains of mice. Tumors were subinoculated into eight inbred mouse strains selected to represent four different alleles at the histocompatibility-2 locus; a difference between the tumor and host at this genetic locus is known to prevent the progressive growth of most transplantable tumors (19).[2]

The results of this experiment are summarized in Table 4. Clone 929 tumor tissue failed to grow in all mice tested except mice of strain $C3H_f$/He; tumor #18 (Table 3) grew in all strain $C3H_f$/He mice injected and tumor #17 in one of ten mice injected. A specificity of clone 929 cells for the strain C3H mouse was thus demonstrated.

TABLE 2

RESULTS OF EXPERIMENTS TO TEST WHETHER CLONE 929 CELLS IMMUNIZE THE STRAIN C3H MOUSE

Experiment no.	Months *in vitro* of clone 929 cells used for immunization	No. tumors from immunizing dose	Results* of test with clone 929 tissue culture cells	
			Immunized	Nonimmunized
1	64	0	0/14	6/16
2	65	0	0/8	7/11
3	67	1	0/9	9/12

* The denominator represents the total number of mice injected and surviving; the numerator represents the number of mice developing sarcomas at the injection site.

TABLE 3

RESULTS OF EXPERIMENT TO TEST WHETHER CLONE 929 TUMOR CELLS CAN BE ADAPTED TO GROW IN 100 PER CENT OF STRAIN C3H MICE INJECTED

Generation *in vivo* of tumor subinoculated	Results of subinoculating tumors into non-irradiated strain C3H mice* Tumor no.																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	0/10		0/10	1/5	3/4	2/5	0/3	1/5	0/4	4/6	1/6	0/4	0/3	1/2				1/5†
2		0/3	2/3				0/8	2/8	1/8							1/6	0/4	2/6†
3			2/10†				7/7	4/8†	1/7						0/7	2/5†	0/5	8/8†
4																2/6†	0/5	4/4†
5															2/5	1/10†	0/3	10/10†
6															11/12†	0/8†		5/5†
7															9/15†			4/4†
8															5/7†			4/4†

* The denominator represents the number of mice inoculated; the numerator represents the number developing sarcomas.
† The tumor subinoculated was taken from a nonirradiated mouse; all other tumors were subinoculated from x-radiated mice.

DISCUSSION

The first finding of the present study was that the clone of cells derived from strain L was still capable of giving rise to a low percentage of sarcomas when injected into strain C3H/He mice. This incidence (15 per cent) was higher than that obtained in 1947 with strain L cells (1 per cent),

[2] The authors wish to express their appreciation to Dr. Lloyd Law of this Institute, who suggested and helped with this phase of the study and supplied the mice used.

but in the present study approximately 6 times the number of cells were injected into each mouse; this increased number of cells may account for the higher percentage of sarcomas produced, since, with tumor strains known to induce resistance in the host, the dosage of injected cells appears to be an important factor in tumor development (9, 13).

The high percentage of sarcomas (68 per cent) developing from clone 929 cells in x-radiated mice suggested that cells of this clone induced a resistance in the nonirradiated host that could be partially abrogated by whole-body x-radiation before cell injection. Further tests confirmed this hypothesis. It was thus demonstrated that clone 929 cells induce an immunity in the strain C3H mouse.

Although generally a tumor derived from a mouse of an adequately inbred strain will grow in all mice of the same strain, a few cases have been reported of immunity induced in inbred strains of animals from tumors autogenous to the strain. Gross (13) demonstrated that a methylcholanthrene-induced sarcoma of strain C3H mice that had been carried for 24 transfers in the strain of origin induced immunity in strain C3H mice. Lewis *et al.* (14) found that sarcomas that had originated in two inbred strains of rats induced an immune state in the strain of origin when the tumors were caused to atrophy and regress by strangulation. MacDowell *et al.* (16) immunized strain C58 mice with lymphatic leukemia cells that had originated spontaneously in this inbred strain many generations previously. Fink (7) demonstrated that, by the 65th generation *in vivo*, a fibrosarcoma induced by methylcholanthrene in a strain BALB/c mouse could immunize mice of this strain. There was some evidence of mutation in the host strain, since the strain of mice in which the tumor originated could be less effectively immunized than a substrain separated about 32 generations prior to tumor origin. Goldfeder (12) has recently reported that a spontaneous mammary adenocarcinoma of the DBA mouse under certain conditions induced demonstrable immunity in the DBA mouse after eight generations *in vivo*. Foley (10) likewise demonstrated immunity of strain C3H mice to spontaneous lymphosarcoma 6-C3H-Ed originating in this strain. In addition, six unselected, methylcholanthrene-induced sarcomas of strain C3H/He mice were found regularly to induce immunity in this strain when the sarcomas were caused to regress as a result of ligation (11); furthermore, immunity could be induced against all sarcomas at either the first or second transplant generations; thus an incompatibility between tumor and host was demonstrated before repeated transplants had been made.

These cases of immunity induced in an inbred strain have been generally interpreted as the result of mutational differentiation of either tumor or stock during the period of transfers (20). Similarly, in the present study, the incompatibility between clone 929 tissue culture cells and the strain C3H mouse has probably resulted from mutational differentiation either in the tissue culture cells or in the host strain C3H mouse during the 13-year period of tissue growth *in vitro*.

Just when the initial change or changes took place is difficult to ascertain. When this strain of cells was first tested in 1942, after 2 years of growth *in vitro* and after exposure to the carcinogen 20-methylcholanthrene (4), only 46 per cent of the mice injected developed sarcomas. Other strains that originated from the same explant and that had been treated with varying doses of methylcholanthrene, when tested in the same groups of mice, gave varying percentages of sarcomas, ranging from 8 per cent in the strains subject to the highest dosages of carcinogen to 86 per cent in the control untreated strains. It is possible that the varying incidence of sarcomas produced by these six cell strains in 1942 already reflected antigenic variations among the tissue culture cell strains.

When in 1943 and 1944 the six cell strains were studied in transparent chambers, only 12 per cent of all 50 transplants grew progressively as tumors; the others either underwent early necrosis or they grew temporarily and later regressed or survived; however, larger implants of entire cultures of these six strains inoculated into the same source of strain C3H mice gave rise at this time to significantly

TABLE 4

RESULTS OF INJECTING CLONE 929 TUMORS INTO VARIOUS INBRED STRAINS OF MICE

Tumor no.	Generation *in vivo* of tumor subinoculated	Results of subinoculating tumors into the following strains of mice*							
		A/L	A/He	BALB/c	DBA/2	C57BR	C3Hf/He	C57BL	C57L
4	1				0/2				
17	7	0/10	0/5	0/7	0/10	0/5	1/10	0/5	0/5
18	4	0/10		0/10	0/10		10/10		
	5		0/10			0/6		0/10	0/10

* The denominator represents the number of mice injected and surviving; the numerator represents the number of mice developing sarcomas.

higher percentages of sarcomas. It appeared that low numbers of cells were not able to grow so readily as larger numbers in the strain C3H mouse. Since, with strains of cells known to induce immunity in the host, the dosage of injected cells is a critical factor in the success of progressive cell growth (9, 13, 16), the failure of these small transplants to grow progressively may have reflected even at this time an incompatibility between strain L cells and the host strain of mouse. In 1948 the first definite indication of an immune reaction in the strain C3H mouse to strain L cells was reported (1).

An additional finding of the present study was that clone 929 cells could be adapted by serial transfer to grow progressively in all strain C3H mice injected. It is not known at the present time whether this adaptation involved the selection of a more malignant or possibly less antigenic sarcoma cell type from a mixed cell population which arose among the progeny of a single cell, or whether further mutational changes occurred in the tumor cells during the serial transfers *in vivo*.

From the tests of this study tumors derived from clone 929 and transplanted from the strain C3H mouse grew progressively only in strain C3H mice; a specificity of clone 929 tumor cells for the strain C3H mouse was thus demonstrated.

SUMMARY

A clone (clone 929) of mouse cells derived in 1948 from a single cell of strain L has been examined for tumor-producing capacity. Strain L originated in 1940 from an explant of subcutaneous connective tissue taken from a strain C3H (Andervont substrain) mouse, and had been cultured *in vitro* in a heterologous culture medium for over 10 years. The incidence of sarcomas produced by this strain had dropped from 68 per cent in 1943 to 1 per cent in 1946.

The first finding of the present study was that clone 929 cells were still capable of giving rise to a low percentage of sarcomas when injected into strain C3H mice. A second finding was that these cells induced an immune reaction in the strain C3H mouse. The development of this incompatibility between tissue culture cells and host strain of origin has been interpreted as due to mutational differentiation of either strain L tissue culture cells or the inbred strain C3H mouse during the 13-year period of tissue growth *in vitro*. A third finding of the present study was that tumors derived from clone 929 could be adapted by serial transfer *in vivo* to grow in all strain C3H mice injected. A specificity of the tumor tissue for the strain C3H mouse was also demonstrated.

REFERENCES

1. Algire, G. H.; Chalkley, H. W.; and Earle, W. R. Vascular Reactions of Normal and Malignant Tissues *in Vivo*. III. Vascular Reactions of Mice to Fibroblasts Treated *in Vitro* with Methylcholanthrene. J. Nat. Cancer Inst., **11**:555–79, 1950.
2. Earle, W. R. Production of Malignancy *in Vitro*. IV. The Mouse Fibroblast Cultures and Changes Seen in the Living Cells. J. Nat. Cancer Inst., **4**:165–212, 1943.
3. Earle, W. R., and Highhouse, F. J. Culture Flasks for Use with Plane Surface Substrate Tissue Cultures. J. Nat. Cancer Inst., **14**:841–51, 1954.
4. Earle, W. R., and Nettleship, A. Production of Malignancy *in Vitro*. V. Results of Injections of Cultures into Mice. J. Nat. Cancer Inst., **4**:213–27, 1943.
5. Earle, W. R.; Shelton, E.; and Schilling, E. L. Production of Malignancy *in Vitro*. XI. Further Results from Reinjection of *in Vitro* Cell Strains into Strain C3H Mice. J. Nat. Cancer Inst., **10**:1105–13, 1950.
6. Evans, V. J.; Earle, W. R.; Sanford, K. K.; Shannon, J. E.; and Waltz, H. K. The Preparation and Handling of Replicate Tissue Cultures for Quantitative Studies. J. Nat. Cancer Inst., **11**:907–27, 1951.
7. Fink, M. A.; Snell, G. D.; and Kelton, D. Demonstration of Antibody in Strain BALB/c Mice to Homologous Tumor S621 by the Use of Two Technics—Anaphylaxis and Tumor Regression. Cancer Research, **13**:666–71, 1953.
8. Fischer, A., and Davidsohn, F. Present Condition of a Twelve-Year-Old Pure Strain of Carcinoma Cells *in Vitro*. Nature, **43**:436–37, 1939.
9. Foley, E. J. Retardation and Regression of Lympho-Sarcoma in C3H Mice Treated with Alien Mouse Spleen and A-Methopterin. Proc. Soc. Exper. Biol. & Med., **79**:155–58, 1952.
10. ———. Immunity of C3H Mice to Lymphosarcoma 6-C3H-Ed Following Regression of the Implanted Tumor. *Ibid.*, **80**:675–77, 1952.
11. ———. Antigenic Properties of Methylcholanthrene-induced Tumors in Mice of the Strain of Origin. Cancer Research, **13**:835–37, 1953.
12. Goldfeder, A. Studies on Radiosensitivity and Immunizing Ability of Mammary Tumours of Mice. Brit. J. Cancer, **8**:320–35, 1954.
13. Gross, L. Intradermal Immunization of C3H Mice against a Sarcoma That Originated in an Animal of the Same Line. Cancer Research, **3**:326–33, 1943.
14. Lewis, M. R.; Maxwell, D. B.; and Aptekman, P. M. Atrophy of Sarcoma in Rats Followed by Tumor Immunity. Surgery, **30**:689–94, 1951.
15. Lewis, W. H. Normal and Malignant Cells. Science, **81**: 545–53, 1935.
16. MacDowell, E. C.; Taylor, M. J.; and Potter, J. S. Immunization of Mice Naturally Susceptible to a Transplantable Leukemia. Proc. Soc. Exper. Biol. & Med., **32**: 84–86, 1934.
17. Murphy, J. B., and Morton, J. J. The Lymphocyte in Natural and Induced Resistance to Transplanted Cancer. II. Studies on Lymphoid Activity. J. Exper. Med., **22**: 204–11, 1915.
18. Sanford, K. K.; Earle, W. R.; and Likely, G. D. The Growth *in Vitro* of Single Isolated Tissue Cells. J. Nat. Cancer Inst., **9**:229–46, 1948.
19. Snell, G. D. The Immunogenetics of Tumor Transplantation. Cancer Research, **12**:543–46, 1952.
20. Snell, G. D.; Cloudman, A. M.; Failor, E.; and Douglass, P. Inhibition and Stimulation of Tumor Homoiotransplants by Prior Injections of Lyophilized Tumor Tissue. J. Nat. Cancer Inst., **6**:303–16, 1946.