CHARACTERIZATION OF L5178Y LEUKEMIC CELLS WHICH RAPIDLY DEVELOP AND LOSE IMPLANTATION ABILITY

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SUMMARY: Two populations of L5178Y murine leukemic cells, maintained by different methods, were studied for their implantation ability in BDF_1 mice. Implantation ability was measured by number of tumor nodules formed, liver weight, and day of death of the animal. 1) Cells from a population grown for 10 years in vitro had no implantation ability; i.e., no tumor nodules were formed when injected into the tail vein. After 30 days of growth in the peritoneal cavity of BDF_1 mice, these same cells were injected into the tail vein and 10 days later had produced over 200 liver tumor nodules. When cells taken from these tumors were recultured for 60 days in vitro, they lost the acquired implantation ability, but regained it after another single peritoneal passage. 2) L5178Y murine leukemic cells grown for six years in ascites tumor cells were extremely tumorigenic; over 200 tumor nodules appeared in the liver after tail vein injection. These cells were not rendered less tumorigenic and did not lose their implantation ability by in vitro culturing for 60 days. The results suggest that implantation ability is a property of the cell's growth environment; furthermore, they have strong implications for the in vivo and in vitro manipulation of this property.

One of the most devastating properties of many primary tumors is their ability to metastasize. Tumor emboli released from the primary tumor invade the vasculature and spread to other parts of the body through the blood stream and lymphatics. Many released cells perish in this transport (1) or remain "sterile" or dormant (2,3) at their site of arrest; however, some go on to grow, escape the host's defense mechanisms, and form secondary metastatic tumors.

One method for studying the fate of tumor emboli after they detach from the primary tumor and invade the blood stream is to inject a tumor cell suspension intravenously; the appearance of tumor nodules after such an injection is a measure of implantation ability (4). By in vitro or in vivo manipulation of the injected cells, a system of rapidly changing, reversible implantation ability can be established, which may elucidate some of the factors that affect the development of successful metastatic foci.

Using this technique, Fidler (5) increased the implantation ability of B16 melanoma in C57Bl/6J mice by selective in vitro culturing of lung tumor nodules formed after an intravenous injection of melanoma cells and using

these in vitro cultured cells for the next sequential in vivo injection. In the present communication we report the development of implantation ability in a cell line that initially lacked it by a single in vivo intraperitoneal transplantation and the complete gradual loss of that acquired implantation ability in 50-60 days through subsequent in vitro cell culturing. Thus, a change in the cell's growth environment, i.e., from in vivo to in vitro growth, vastly altered the implantation potential of a cell line.

MATERIALS AND METHODS

Cells and cell maintenance. Two populations of L5178Y murine leukemic cells were used: 1) Cells cultured in vitro for 10 years (6). These cells, referred to here as L5178Y/C, were maintained in culture in Fischer's medium with 10% horse serum (both purchased from Grand Island Biological, Grand Island, N.Y.). 2) Cells grown as ascites tumors for 6 years and maintained by weekly intraperitoneal injection of 0.2 ml of L5178Y cells containing ascites fluid in DBA/2 x C57B1/6 (BDF_1) mice (Jackson Laboratory, Bar Harbor, Maine). These cells are referred to as L5178Y/ascites.

Ascites conversion of L5178Y/C cells. To convert the L5178Y/C cells to the ascites form, a peritoneal transfer of 10^6 cells/0.2 ml of Fischer's medium in a single cell suspension was made. These cells were harvested and then placed back in culture. Cells handled in this manner are referred to as $L5178Y/CAC_n$, where n is the number of days the cells have been in culture. This process can be repeated (i.e., after in vitro culture the cells can be grown again as ascitic cells), yielding $L5178Y/CAC_{90}AC_m$(i.e., cells harvested from the first peritoneal transfer, kept for 90 days in cell culture, returned to the peritoneal cavity of an animal, reharvested from the resulting ascites tumor, and cultured for a further m days). Growth time in vivo as an ascites tumor is fairly constant, but the number of days of culture in vitro varies in the experiments described. Using this nomenclature, $L5178Y/CAC_0$ refers to cells that after ascites growth were not grown in cell culture in vitro.

Implantation assay. The implantation assay was performed by an intravenous injection of 10^5 cells/0.2 ml of Fischer's medium in the tail vein of a BDF_1 mouse. Except where otherwise indicated, the mice were killed by cervical dislocation 10 days after the injection. Tumor formation was measured by three methods: a) number of tumor nodules in the indicated organ, b) weight of tumor-invaded organ, expressed as percent of body weight (in cases of large numbers of tumor nodules the extent of invasion is so great that number of nodules alone is not a sufficient measure), and c) day of death due to tumor invasion.

RESULTS

Initial observations. L5178Y/C cells (cells always grown in in vitro cell culture) had no implantation ability; i.e., when they were injected into the tail vein of BDF_1 mice, no tumors were formed. Extending the time period of the implantation assay of the injected cells from the usual 10 days to 15-56 days still did not result in tumor formation. Moreover, five mice kept for observation still appeared healthy after five months.

L5178Y/ascites cells (cells always grown in vivo in the peritoneal

cavity) were found to be extremely tumorigenic. Large numbers of tumors were found, and the animals usually died from the tumor load on day 10 or earlier. Examination of the animals after death showed that the liver had the highest number of tumor nodules. In each of the 30 mice used in this experiment, more than 200 tumor nodules were counted in the liver, and liver weight was increased three- to fourfold over normal. Some tumors were found in the kidney but none in the lungs.

Development of implantation ability. L5178Y/C cells were converted to the ascites form, L5178Y/CAC_0, by a single intraperitoneal injection. Injected mice died of ascites tumors in about 30 days. Tumor cells harvested from the peritoneal cavity of these mice after the maximum time in vivo were tested for their implantation ability. They were found to possess a high implantation ability, about equal, in fact, to that of L5178Y/ascites cells. (See Figure 1.) Thus, L5178Y/C cells, which had zero implantation ability, were transformed to L5178Y/CAC_0 cells with extremely high implantation ability.

Loss of implantation ability. L5178Y/CAC_0 cells were cultured under the same conditions as are routinely used to grow L5178Y/C cells and were found to have a similar generation time as the L5178Y/C cell line--9.5 to 10 hours. Cells from this culture were tested at various times (L5178Y/CAC_n) for their implantation ability. As can be noted in Figure 1, there was a drastic reduction of implantation ability after 15 days in cell culture and a complete loss by 60 days. Thus, L5178Y/CAC_0, which had extremely high implantation ability, lost it (in a direct temporal relationship) completely by 60 days in in vitro cell culture.

Reversibility of loss of implantation ability. L5178Y/CAC_{94} cells, after a single intraperitoneal passage, were converted to ascites form which was able to kill 5 injected mice in 28.0 ± 0.8 days (compared to less than 10 days for L5178Y/ascites and 20-40 days for L5178Y/C) when administered by intraperitoneal injection. Tumor cells harvested from one of these mice at death, i.e. after maximum time (36 days) in vivo, designated as L5178Y/-$CAC_{94}AC_m$, were cultured in vitro with the following results: a) After two days in culture, high implantation ability was still present; death of the mice occurred in 18.0 ± 0.8 days, with over 300 tumor nodules in the liver and a fourfold increase in liver weight (23.1 ± 2.1% of body weight). b) After 44 days in culture, very low implantation ability was noted. By normal assay, implantation appeared to be zero, but the two mice held for observation died, one at 18 days and one at 21 days after tail vein injection. The first had extensive tumor invasion, including 3 or 4

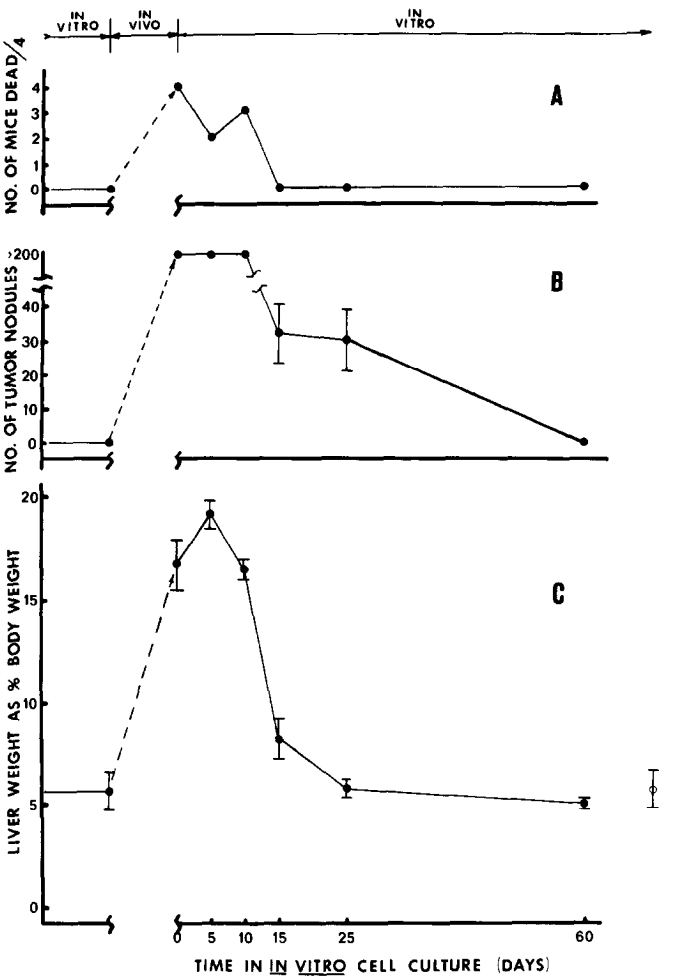

Fig. 1. The implantation ability of L5178Y/C, L5178Y/CAC_0 and L5178Y/CAC_n cells. (Subscript n refers to number of days in cell culture.) L5178Y/C cells (cells always grown in cell culture) are shown on the left of the broken line of the time (y) axis and under the first in vitro arrow located at the top of the figure. 10^6 L5178Y/C cells were injected intraperitoneally, causing an ascites tumor (indicated by the broken part of the line on the time axis and under the in vivo arrow at the top of the figure). Cells were then harvested from the peritoneal cavity of a mouse dying from the ascites tumor; these cells are designated L5178Y/CAC_0 (see text) and are indicated by the first time point (time 0) at the right of the broken line on the time axis and under the second in vitro arrow at the top of the figure. The harvested cells were placed in complete Fischer's medium at 10^5 to 5 x 10^5 cells/ml for n days (with the medium changed daily)--L5178Y/-CAC_n.

Implantation ability was measured by injection of 10^5 tumor cells into the tail vein of each of 4 BDF_1 mice. Ten days after the injection, the mice were killed and examined. A: number of mice dead at 10 days divided by the total number of mice (4) at each point. B: average number of tumor nodules at the surface of the liver ± S.E.M. C: liver weight expressed as percent of body weight ± S.E.M. Normal liver weight is shown by an open circle on the right of the graph.

large liver nodules, increased liver weight (16.4% of body weight), and two large nodules replacing each kidney. The other mouse had no apparent tumors and its weight was significantly lower than normal, an indication that death was not due to tumor since tumor-bearing animals maintain normal weight. c) After 73 days in in vitro cell culture, no tumor formation was found in the mice 67 days after tail vein tumor cell injection in the normal implantation assay.

Failure to alter implantation ability of L5178Y/ascites cells by culturing in vitro. L5178Y/ascites cells were placed in cell culture and had a generation time of approximately 12 hours. They had a great tendency to agglutinate, in contrast to L5178Y/C, which always grow in vitro as a single cell suspension. L5178Y/ascites cells were tested for implantation ability after various times in culture. The results are given in Figure 2. There was no significant change in the implantation ability of L5178Y/ascites cells during the 60 days in in vitro cell culture.

DISCUSSION

The procedure of growing cells of the L5178Y/C leukemia cell line alternately in vivo and in vitro establishes a system in which implantation ability can be monitored and manipulated. The establishment of this system of gain and loss of implantation ability extends the observation of Fidler (5) that metastatic potential lies partially in properties unique to the tumor cell population. However, one cannot ignore the host, since the cells lodge and grow in the liver and randomly in the kidneys after passage through the lungs. The system demonstrates the ability of a cell population to change the properties that determine metastatic potential, either by a reversible selection of preexisting clones that have various metastatic potentials or by the adaptation of the entire cell population to the various growth conditions imposed upon it.

The present system also demonstrates that the route of injection used to test tumorigenicity is critical, since the L5178Y/C cells were tumorigenic

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Fig.1 contd.

For example, L5178Y/C produced zero tumor nodules (B) under the implantation assay conditions, but when placed in vivo (10^6 cells, i.p.) they were transformed to L5178Y/CAC_0 at harvest, which produced over 200 tumor nodules in the liver under the implantation assay conditions (see text). After 5 days in culture, 2 out of 4 mice were dead after 10 days (A), still more tumor nodules appeared in the liver (B), causing the liver weight to be 18.3 ± 0.6% of body weight (C) (normal, 5.7 ± 0.9%).

by the intraperitoneal route but nontumorigenic by the intravenous route. It is also evident from the results presented that the method of maintaining the cell line can introduce vast differences in implantation ability, as exemplified by L5178Y/C vs L5178Y/ascites.

Two reports (7,8) demonstrating alteration of cell population characteristics have a bearing on the present system, although they described nontypical *in vitro* growth conditions leading to systems not amenable to biochemical analysis. Smets et al. (7), studying MB VIA lymphosarcoma cells, observed a gain of implantation ability after one passage *in vivo* and a subsequent rapid loss (sometimes in one day) of this implantation ability upon *in vitro* culturing by seeding the cells in culture medium at 10^7 cells/ml. Since the L5178Y/C cells enter into stationary phase growth at 2×10^6 cells/ml, such a high cell concentration could not be attained in our system; hence, the cell concentration was kept between 10^5 and 5×10^5 cells/ml by daily medium change. Another alteration of the tumor population by *in vitro* culturing under minimal growth or nongrowth conditions was performed by Parks (8), who showed that the ability of S-91 mouse melanoma to metastasize *increases* after 16 days in *in vitro* cell culture.

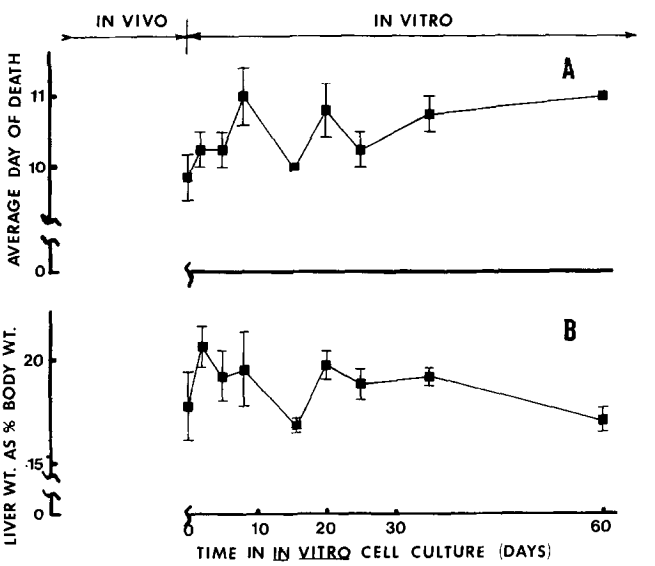

Fig. 2. The effect of *in vitro* culturing on implantation ability of L5178Y/ascites tumor cells. These cells, derived from L5178Y cells carried by intraperitoneal animal passage in ascites form for six years, were cultured in complete Fischer's medium. Implantation ability was measured as in Fig. 1, except that tumorigenicity was determined by average day of death ± S.E.M. (A) and average liver weight as percent of body weight ± S.E.M. (B).

Sanford (9) had shown earlier that under maximum proliferative growth conditions, the increase in tumor-producing capacity produced by an in vivo passage of a clone of cells isolated from mouse L cells subsides after 8 months in culture. The timing and growth patterns of the present system allows the study of the biochemical parameters that change in accordance with the implantation changes in order to relate a biochemical event to a whole body responses, i.e. the ability of a tumor embolus to form a metastasis.

Bosmann et al. (10), using as a basis the system Fidler (5) developed, found that the clone of cells that showed high implantation ability had higher electrophoretic mobility and greater synthetic and degradative enzymatic activities than the clone of cells that showed low implantation ability when both were grown under sparse culture conditions. Similar experiments are planned in which biochemical parameters will be measured in order to relate them to the reversible gain and loss of tumorigenicity. Such correlations will then be used to examine a number of cellular factors postulated to influence the host-tumor cell interaction: altered antigenicity (11-14). rate of membrane shedding (15), release of blocking factors (16), and other possible subversions of the immune system (17).

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REFERENCES

1. Fidler, I. J. (1970) J. Nat. Cancer Inst. 45, 773-782.
2. Fisher, E. R. and Fisher, B. (1965) Acta Cytol. 9, 146-159.
3. Eccles, S. A. and Alexander, P. (1975) Nature 257, 52-53.
4. Willis, R. A. (1973) The Spread of Tumors in the Human Body, 3d. ed. Butterworth and Co., Ltd., London.
5. Fidler, I. J. (1973) Nature New Biol. 242, 148-149.
6. Fischer, G. A. (1958-9) Ann. N.Y. Acad. Sci. 76, 673-680.
7. Smets, L. A. and Broekhuysen-Davies, J. (1972) Eur. J. Cancer 8, 541-548.
8. Parks, R. C. (1975) J. Nat. Cancer Inst. 54, 1473-1474.
9. Sanford, K. K., Merwin, R. M., Hobbs, G. L. and Earle, W. R. (1959) J. Nat. Cancer Inst. 23, 1061-1070.
10. Bosmann, H. B., Bieber, G. F., Brown, A. E., Case, K. R., Gersten, D. M., Kimmerer, T. W., and Lione, A. (1973) Nature 245, 487-489.
11. Axelrad, A. and Klein, G. (1956) Transpl. Bull. 3, 100-102.
12. Ioachim, H. L., Keller, S. E., Dorsett, and Pearse, A. (1974) J. Exp. Med. 139, 1382-1394.
13. Sugarbaker, E. V. and Cohen, A. M. (1972) Surgery 72, 155-161.
14. Cikes, M., Friberg, S. and Klein, G. (1973) J. Nat. Cancer Inst. 50: 347-362.
15. Alexander, P. (1974) Cancer Res. 34, 2077-2082.
16. Hellstrom, I. and Hellstrom, K. E. (1970) Inst. J. Cancer 5, 195-201.
17. Plescia, O. J., Smith, A. H., and Grinwick, K. (1975) Proc. Nat. Acad. Sci. USA 72, 1848-1851.