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Spontaneous Disappearance of Tumorigenicity in a Polyoma Virus-Induced Neoplasm Carried *in Vitro* (37815)

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Tumors induced by polyoma virus have been found to be heterogeneous in expression of several properties. Rabinowitz and Sachs (1–3) reported that once polyoma transformation had become a hereditary cellular property, a high frequency of variants occurred with reversion towards normal of properties characteristic of transformation without loss of the viral genome detected by the presence of a nuclear T antigen. Hare (4) reported increasing difficulty in transplantation of polyoma-induced tumors in hamsters after 90 passages *in vitro*. Walls and Negroni (5, 6) studied cloned polyoma cell lines from C3H mice and found one clone whose morphologic and tumorigenic properties differed from those of other clones. This particular clone was not transplantable in syngeneic recipients despite pretreatment with X-irradiation and cortisone. This cell line was capable, however, of inducing transplantation resistance to other tumorigenic polyoma cell lines. The present report describes the spontaneous disappearance of tumorigenicity and some of the characteristics of a tissue culture-passaged cell line established from a polyoma-induced mouse tumor.

Methods. Py-89 is a polyoma-induced neoplasm in C57B1/Ka mice originally described by Ting (7). This cell line had been passaged in C57B1/Ka mice and had been adapted to grow in tissue culture in Ham's F-12 medium supplemented with 10% fetal calf serum. Periodic determinations of tumorigenicity were made in syngeneic recipients by injecting from 10^3 to 6×10^7 cells subcutaneously and monitoring the mice for tumor growth.

A ^{51}chromium-release cell-mediated cytotoxicity assay was used to detect H-2^b alloantigens (8). A/J(H-2^a) mice (Jackson Labs, Bar Harbor, ME) received one ip injection of 2×10^7 Py-8914b cells (variant line described in this report). Ten days after immunization, peritoneal exudate cells (PEC) were harvested after one ip injection of 3 ml of sterile sodium caseinate (12% in normal saline, Difco) 12–15 hr prior to harvest. The PEC were washed and incubated with ^{51}Cr-labeled RBL-5 (H-2^b) and YAA-Cl (H-2^a) target cells in RPMI-1640 medium with 20% fetal calf serum (FCS), penicillin (10 U/ml), streptomycin (10 μg/ml) at 37° on a rocking platform (6 rocks/min) for 4.5 hr in 10 × 75 mm glass tubes. B10.D2 (H-2^d) mice were immunized once with 10^7 Sarcoma-I (H-2^a) cells intraperitoneally, and their PEC were tested against both target cells as a specificity control. Effector to target cell ratio was held constant at 200:1. Cytotoxic percentage was determined by subtracting the counts per minute released by normal PEC from cpm released by sensitized PEC divided by the maximum possible release as determined by freeze thawing the target cells four times.

The microcytotoxicity assay of the Hellstroms (9) was used to study tumor-specific antigens on the Py-8914b cell line. C57B1/Ka mice were immunized to polyoma-specific antigens in three ways: 0.1 ml of polyoma virus (Dulbecco's large plaque, 3.6×10^8 PFU/ml donated by Dr. K. Chang) was injected intraperitoneally three times at weekly intervals; 2×10^7 Py-8914b cells were injected intraperitoneally once; animals bearing progressively growing Py-89

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tumors of 10-mm diam or less. Target cells were Py-8914b, MCA 8/2(3-methylcholanthrene-induced tumor in C57B1/Ka mice), and SSA-4 (spontaneous sarcoma in C57B1/Ka mice donated by Dr. M. Lieberman of Stanford University). Sixty target cells were seeded into each well of Falcon microtest-II platss in Ham's F-12 medium with 10% FCS. After the target cells attached to the wells, medium was removed and lymph node lymphocytes (1.5×10^5/well) which had been minced through a wire screen were added to each well in Ham's F-12 without FCS. The plates were incubated for 45 min at 37° in CO_2 after which 0.05 ml of F-12 with 50% FCS was added. Plates were incubated for 40 hr at 37° in CO_2, and nonadherent cells were washed from the wells using normal saline with 10% FCS. Adherent cells were fixed with methanol and stained with Giemsa and crystal violet. The stained adherent cells were counted without knowledge of the experimental protocol using a dissecting microscope.

Results. After 100 passages in tissue culture, Py-89 cells spontaneously lost their ability to cause tumors in syngeneic recipients even when 6×10^7 cells were given intraperitoneally. Previously, as few as 10^3 cells would produce tumors in 50% of syngeneic recipients. The *in vitro* growth pattern had changed. The individual cells retained their elongated fibroblastic appearance, but they now grew in dense sheets whereas previously they had grown in a "swiss-cheese pattern" with much piling up of cells, leaving large areas free of cells. This variant cell line was designated Py-8914b to distinguish it from other Py-89 cell lines that remained tumorigenic. Since our original observation, two other cell lines of explanted Py-89 cells have similarly lost their ability to cause tumors after from 90 to 110

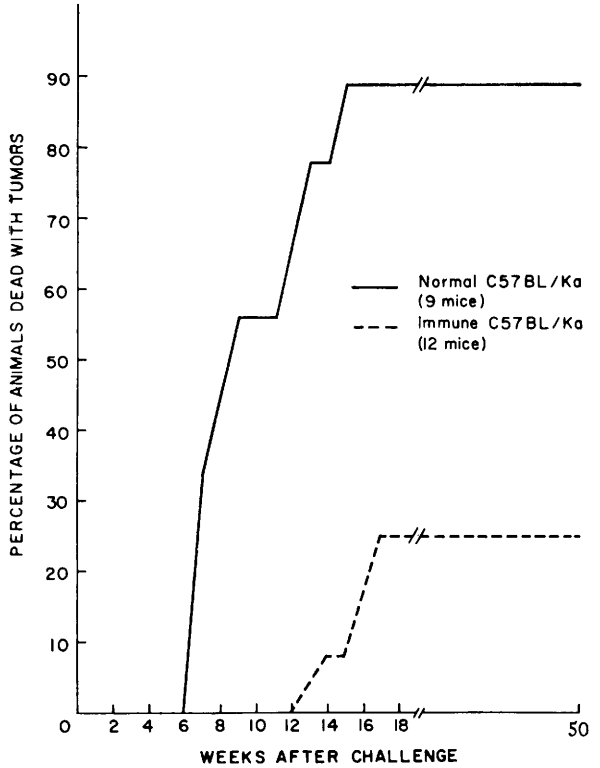

FIG. 1. Induction of syngeneic transplantation immunity to Py-89 fibrosarcoma in C57B1/Ka mice by immunization with five injections of 2×10^6 Py-8914b cells at weekly intervals. Normal mice consisted of nine animals (solid line) and immune group consisted of 12 mice (broken line). All mice were challenged with 10^5 Py-89 cells 2 weeks after last immunization.

TABLE I. Demonstration of Specific H-2 Alloantigens on Py-8914b Cells Using a ^{51}Cr-Release Cell-Mediated Cytotoxicity Assay.

Effector Cells	RBL-5 (H-2^b)	YAA-Cl (H-2^a)
A/J anti-Py-8914b	43.0 ± 4.1[a,c]	2.4 ± 1.5[b]
B10.D2 anti Sarcoma-I	−1.1 ± 2.7[b]	15.8 ± 2.0[c]

[a] Cytotoxic percentages ± 1 SEM.
[b] Not statistically significant.
[c] Significant at $P = .001$.

passages *in vitro*. Both of these lines also exhibited a change to a growth pattern resembling that of Py-8914b.

Five weekly sc injections of 2×10^6 Py-8914b cells into C57Bl/Ka mice induced transplantation resistance to various challenge doses (10^4, 10^5, 10^6) of tumorigenic Py-89 cells. In Fig. 1, the immunized mice were protected from challenge as only 3 of 12 died from tumors whereas 8 of 9 untreated mice died from similar challenge ($P < .003$).

Py-8914b cells were capable of specifically sensitizing PEC to lyse H-2^b target cells in the ^{51}Cr-release cell-mediated cytotoxicity assay (Table I), thereby demonstrating the retention of their original alloantigens.

Lymphocytes sensitized to polyoma-specific antigens killed Py-8914b cells in the microcytotoxicity assay (Table II) but did not kill the antigenically unrelated SSA-4 cells in two representative experiments. Sixteen separate experiments were done to determine the effect of polyoma-sensitized lymphocytes on both Py-8914b and MCA 8/2. In 2 representative experiments (Table II) polyoma sensitized lymphocytes significantly killed both Py-8914b and MCA 8/2 cells. Py-8914b cells, however, were killed to a significantly greater degree (52.2%) than MCA 8/2 (30.4%, $P < .01$).

Discussion. The spontaneous disappearance of tumorigenicity in an *in vitro* passaged by Py-89 cell line was accompanied by changes in *in vitro* growth pattern as described. This change in tumorigenicity and growth pattern was repeatable in that it occurred twice in subsequently explanted and passaged cell lines which originated from the parent Py-89 cell line.

This variant cell line was capable of inducing transplantation resistance to other Py-89 cell lines while retaining its original alloantigen. Lymphocytes sensitized in various ways to polyoma antigens killed Py-8914b and not SSA-4 in the microcytotoxicity assay thereby demonstrating *in vitro* detectable tumor-specific antigens. An apparent antigenic cross-reactivity was detected between polyoma antigens and those of the MCA 8/2 *in vitro*. Prehn (10) and Braun (11) have described a possible antigenic relationship between mouse embryo cells

TABLE II. Results of Microcytotoxicity Assay Using Py-8914b, SSA-4, and MCA 8/2 Target Cells.[a]

Effector Cells	Py-8914b Adherent Cells + SEM	% Reduction	SSA-4 Adherent Cells + SEM	% Reduction
Expt. 1. Nonimmune	90 ± 4.4		82 ± 4.8	
Immune[b]	51 ± 3.4	43.3 (P .001)	73 ± 5.0	10.0
Expt. 2. Nonimmune	37 ± 2.6		196 ± 13.2	
Immune[b]	28 ± 3.2	24.3 (P .05)	172 ± 12.2	
	Py-8914b Adherent Cells + SEM	% Reduction	MCA 8/2 Adherent Cells + SEM	% Reduction
Expt. 1. Nonimmune	46 ± 5.0		90 ± 7.0	
Immune[b]	20 ± 1.8	57.0 (P .005)	62 ± 2.8	31.0 (P .01)
Expt. 2. Nonimmune	121 ± 9.1		26 ± 2.5	
Immune[b]	58 ± 3.1	52.0 (P .0005)	18 ± 3.0	31.0 (P .05)

[a] Average percent reduction for 16 experiments using Py-8914b and MCA 8/2 targets. Py-8914b = 52.2 ± 3.7; MCA 8/2 = 30.4 ± 5.7 ($P < .01$).
[b] Lymphoid effector cells specifically sensitized to polyoma antigens.

and tumors induced by 3-methylcholanthrene in mice. In addition, Pearson and Freeman (12) described antigenic cross-reactivity between polyoma transformed hamster cells and 12-day-old hamster embryo cells. It is possible that there is some basis for the apparent antigenic cross-reactivity demonstrated *in vitro* between Py-8914b and MCA 8/2 in that both lines may be antigenically related to similar cells, namely mouse embryo cells, and the cross-reactivity may be recognition of embryonic membrane antigens.

Summary. A polyoma-induced cell line which spontaneously lost its tumor-causing capability while being passaged *in vitro* was described. This cell line retained both its appropriate H-2 alloantigen and its viral-related tumor antigen of transplantation type. The fortuitious observation of *in vitro* cross-reactivity between this cell line and a syngeneic 3-methylcholanthrene-induced tumor is discussed.

1. Rabinowitz, Z., and Sachs, L., Nature **220**, 1203 (1968).

2. Rabinowitz, Z., and Sachs, L., Virology **38**, 336 (1969).

3. Rabinowitz, Z., and Sachs, L., Virology **38**, 343 (1969).

4. Hare, J. D., and Godal, T., Proc. Soc. Exp. Biol. Med. **119**, 632 (1965).

5. Walls, E. J., and Negroni, G., Eur. J. Cancer **2**, 221 (1966).

6. Negroni, G., and Tilly, R., J. Cell Sci. **7**, 711 (1970).

7. Ting, R. C., Nature **211**, 1000 (1966).

8. Berke, G., Sullivan, K. A., and Amos, D. B., J. Exp. Med. **135**, 1334 (1972).

9. Hellstrom, I., Hellstrom, K. E., Sjogren, H. O., and Warner, G. A., Int. J. Cancer **7**, 1 (1971).

10. Prehn, R. T., *in* "Cross Reacting Antigens and Neoantigens" (J. J. Trentin, ed.), p. 105, Williams Wilkins Co., Baltimore (1967).

11. Braun, R. J., Int. J. Cancer **6**, 245 (1970).

12. Pearson, G., and Freeman, G., Cancer Res. **28**, 1665 (1968).

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