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Quantitative Tumorigenicity Assays Using the Anterior Chamber of the Mouse Eye[1]

Charles W. Boone and Lartius T. DuPree

Cell Biology Section, Viral Carcinogenesis Branch, National Cancer Institute, NIH, USPHS, Bethesda, Maryland 20014

SUMMARY

Using a quantitative technic for depositing a definite number of carcinoma or sarcoma cells into the anterior chamber of the mouse eye, without leakage as proven by complete retention of Trypan blue dye, this route generally required a 100- to 1,000-fold lower dose of cells to produce tumors in 100% of animals, as compared to the intramuscular, intravenous, and intraperitoneal routes of inoculation in both irradiated and nonirradiated mice. Neoplastic transformation of a cultured mouse cell line was demonstrated using the quantitative anterior chamber technic.

INTRODUCTION

Evans *et al.* (2) have demonstrated the utility of the intraocular route in tumorigenicity testing of cultured mouse cell lines. She instilled masses of aggregated cells scraped from the floor of the culture vessel into the anterior chamber of the mouse eye by means of a 0.3 mm diameter eye pipet, based on a procedure described by Grobstein (3). Liebelt and Liebelt (4) describe a similar technic for transplanting solid fragments of normal or neoplastic tissue into the anterior chamber of the mouse eye. We have devised a quantitative technic for depositing a definite number of single cells in suspension into the anterior chamber, thus extending to this route a quantitative accuracy and precision similar to the more conventional routes of inoculation.

To evaluate the comparative efficacy of the anterior chamber route, we determined dose-tumorigenicity curves for carcinoma and sarcoma cells inoculated into the anterior chamber, and compared them with curves using the intramuscular, intravenous, and intraperitoneal routes in both irradiated and nonirradiated mice. A decided shift of the dose-tumorigenicity curve to lower cell doses occurred when the anterior chamber route was used. The details of the tumorigenicity assays by different routes of inoculation, a description of the technic for inoculating a known number of cells into the anterior chamber of the mouse eye, and the demonstration of spontaneous neoplastic transformation in a cultured mouse line through the use of the quantitative anterior chamber technic, form the subject of this report.

MATERIALS AND METHODS

Tumor Cells

The Ehrlich-Lettré ascites carcinoma, which is hyperdiploid (model number of chromosomes = 44) was obtained from Professor Ralph McKee, UCLA Center for Health Sciences, Los Angeles, California. The Sanford 2472 fibrosarcoma was obtained from the American Type Culture Collection, Rockville, Maryland.

Cultured Syngeneic Mouse Cells

Cell line WMS was grown from minced BALB/c weanling mouse spleen using NCTC 109 and 10% fetal calf serum. Circular culture flasks with optically flat surfaces and a bottom surface area of 150 sq cm were used (Corning Glass Works, Corning, New York). As soon as a confluent monolayer formed, the cells were trypsinized and split 1:2. The time to confluency after a split averaged three days. Cells from the first, fifth, eighth, and tenth 1:2 splits were inoculated into the anterior chamber of groups of 25 mice each, 200,000 cells per inoculation.

Mice

Weanling BALB/cAnN males were used for the Ehrlich-Lettré ascites carcinoma inoculations and for the anterior chamber inoculation of cultured syngeneic mouse cells. C3H males were used for the Sanford 2472 fibrosarcoma inoculations. Both strains of mice were obtained from the Division of Research Services, NIH. Following tumor cell inoculation by all routes, mice were scored for tumor growth at thirty days.

Intraocular Inoculation Procedure

1. The mouse is anesthetized with 0.65 mg of Nembutal given subcutaneously.

2. The mouse eye is visualized under the low power of a dissecting microscope, and a 30° incision is made at the corneoscleral junction, parallel to and just anterior to the iris, using a #11 Bard-Parker scalpel blade, as shown in Fig. 1*a*.

3. The aqueous humor is expressed from the anterior chamber by compressing the cornea with the back of the scalpel blade, and the area is blotted dry with a sponge. This step is

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important: it provides space for the fluid volume of the cell inoculum to be injected.

4. The needle of a Hamilton one-microliter gas chromatography syringe (The Hamilton Company, Inc., Whittier, California) is introduced into the anterior chamber through the incision, and the tumor cells in suspension are injected in a volume of 0.5 microliter as shown in Fig. 1*b*. In the figure, Trypan blue is being injected to demonstrate absence of leakage.

5. The needle is withdrawn slowly and steadily with rotation. Leakage of the injected material is prevented by prolapse of the iris, which follows the needle as it is withdrawn and plugs the incision (Fig. 1*c*). The technic is not difficult and can be done rapidly by one operator.

Preparation of Tumor Cell Suspensions for Intraocular Inoculation

5×10^6 viable cells, as determined by Trypan blue dye exclusion, are placed in a 10-ml centrifuge tube possessing a thin stem at the bottom which is graduated in increments of 0.005 ml (Scientific Glassware Apparatus Co., Inc., Bloomfield, N.J.). The cells are centrifuged in an IEC centrifuge Model PR-2 at 1500 rpm for 5 minutes. The cell pack is resuspended in 0.01 ml giving a concentration of 500,000 cells per microliter. Thus, 250,000 cells can be inoculated into the anterior chamber in a volume of 0.5 microliter. Smaller doses of cells per 0.5 microliter are made by dilution. As a check on each dilution, the dose of cells in 0.5 microliter is delivered onto a hemocytometer grid, covered with the coverslip, and the cells counted. The delivery error of the microliter syringe was determined by counting the cells in 50 consecutive deliveries of 0.5 microliter from a suspension of cells. The standard deviation from a mean count of 100.3 cells per delivery was 21.8%.

Irradiation. A dose of 250 R was delivered to mice in groups of 5, four to six hours before inoculation. A higher dose of 300 R was tried but resulted in the death of about 20% of animals after five to fourteen days.

Observation of Animals. For all routes of inoculation mice were scored for tumor growth after thirty days. At this time the smallest intraocular tumor was over two times the size of the eyeball. The site of intramuscular inoculations was sectioned, and the presence of tumor scored macroscopically. The smallest tumors were 0.5 cm, and the largest 2.5 cm, in average dimension. The presence of ascites tumor following intraperitoneal inoculation of Ehrlich-Lettré ascites carcinoma cells was scored grossly and checked by observing tumor cells in the ascitic fluid microscopically. The mice inoculated intravenously were scored macroscopically for pulmonary tumor nodules. For all routes of inoculation except intraperitoneal, the presence of tumor was verified by microscopic examination of representative histologic sections prepared and stained with hematoxylin and eosin by routine methods.

RESULTS

Ehrlich-Lettré Ascites Carcinoma. By the anterior chamber route, only 10 cells were required to produce tumors in 100% of animals, as compared to 10^4 cells by the intramuscular and intravenous routes in irradiated animals and 10^6 cells by the intraperitoneal route in nonirradiated animals (Table 1).

Sanford 2472 Fibrosarcoma. 10^3 cells were sufficient to produce tumor in 100% of animals by the anterior chamber route, as compared to 10^4 cells by the intramuscular route in irradiated mice, and 10^6 cells by the intramuscular route in nonirradiated mice (Table 2).

Effect of Irradiation. In the case of the Sanford fibrosarcoma, reducing the immunologic response of the host mouse by irradiation did not lower the minimum tumorigenic dose when the anterior chamber route was used. This is consistent with the known sequestration of the anterior chamber from immunologic responses. As expected, the minimum tumorigenic dose by the intramuscular route was lowered in irradiated mice for both the Sanford fibrosarcoma and the Ehrlich carcinoma. The marked effect of irradiation in increasing the number of pulmonary tumors following the intravenous inoculation of Ehrlich carcinoma is noteworthy.

Mouse Line WMS (Weanling Mouse Spleen). One of the 25 mice inoculated in the anterior chamber with 200,000 cells from the eighth 1:2 split of line WMS developed a rapidly growing tumor of the eye at the site of inoculation 282 days

Table 1

Nonirradiated mice		Irradiated mice	
Cell dose	Tumorous mice / Total mice	Cell dose	Tumorous mice / Total mice
Intraocular			
125,000	5/5	20,000	6/6
25,000	5/5	6,000	5/5
12,000	5/5	2,000	6/6
1,000	5/5	1,000	6/6
100	5/5	600	5/5
10	5/5	100	5/5
		10	5/5
Intramuscular			
10^7	5/5	10^7	4/4
10^6	5/5	10^6	4/4
10^5	5/5	10^5	4/4
10^4	5/5	10^4	4/4
10^3	0/5	10^3	3/5
10^2	0/5	10^2	1/5
10^1	0/4	10^1	0/5
Intravenous			
10^6	0/5	10^6	1/1[a]
10^5	0/5	10^5	2/2
10^4	0/9	10^4	4/4
10^3	0/7	10^3	3/8
10^2	0/5	10^2	0/3
Intraperitoneal			
10^7	5/5	10^7	not done
10^6	5/5	10^6	not done
10^5	1/4	10^5	3/5
10^4	3/5	10^4	2/5
10^2	0/5	10^2	0/5

Tumorigenicity study, various routes of inoculation, and Ehrlich ascites carcinoma in BALB/c mice (all tumors within 30 days).
[a] Pulmonary tumors at 30 days.

Table 2

Nonirradiated mice		Irradiated, mice	
Cell dose	Tumorous mice / Total mice	Cell dose	Tumorous mice / Total mice
Intraocular			
250,000	5/5	250,000	5/5
25,000	5/5	25,000	5/5
2,500	4/5	2,500	4/4
1,000	5/5	1,000	6/6
500	6/9	500	2/5
100	4/9	100	3/5
10	2/5	10	1/4
Intramuscular			
10^7	5/5	10^7	5/5
10^6	5/5	10^6	5/5
10^5	4/5	10^5	5/5
10^4	7/10	10^4	10/10
10^3	0/5	10^3	1/5
10^2	0/5	10^2	0/5
Intravenous			
10^7	1/3	10^7	0/2
10^6	0/5	10^6	0/5
10^5	0/5	10^5	0/5
10^4	0/6	10^4	0/7
10^3	0/3	10^3	0/4
10^2	0/3		

Tumorigenicity study and various routes of inoculation of high tumor-producing line 2472 (Sanford) in C3H mice (all tumors within 30 days).

after inoculation. It was approximately 1 cm in average diameter at the time of excision. The tumor was subsequently transplanted through 3 mouse passages by subcutaneous inoculation, and then explanted into cell culture and carried for 5 subcultures before being stored viable-frozen. Histologically, the tumor was a typical undifferentiated fibrosarcoma. Anterior chamber inoculation of less than 10,000 cells from the third subculture resulted in a tumor which produced enlargement of the eye within three weeks.

DISCUSSION

The quantitative technic described for instilling a definite number of cells into the anterior chamber represents an improvement over previously described technics in which solid pieces of tissue have been used. Dose-tumorigenicity assays using this route are now possible and at generally lower cell doses so that statistically adequate assays can be performed without the inconveniently large number of cells frequently required when the intramuscular route is used. The anterior chamber route has a number of other unique advantages: tumor growth can be followed from its initial stages by direct inspection through the cornea using dissecting microscope; tissue for histologic sections is simply prepared by enucleating the eye and placing it in formalin; and finally, reproducible tumor growth patterns can be seen histologically against the standard background of the internal structure of the eye. A report by Browning and White (1) illustrates the high degree of morphologic detail which can be seen with a dissecting microscope in tissue which is growing in the anterior chamber of the mouse eye.

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Fig. 1. Technic of depositing a definite number of cells in suspension into the anterior chamber of the mouse eye. *a*, incision at corneoscleral junction just anterior to iris; *b*, instillation of cell suspension using a one-microliter syringe (in the photograph Trypan blue dye is being injected to demonstrate absence of leakage); *c*, after withdrawal of the needle with rotation, the iris prolapses into the incision completely plugging it. It is important to express the aqueous humor from the anterior chamber after the incision so that space is provided for the cell inoculum.