

Loss of Transplantability and Induction of Immunoprotection by Mouse Ascites Tumor Cells in Tissue Culture (34782)

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(Introduced by L. B. Jaques)

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The pioneer experiments of Gey in 1941 (1) and of Earle in 1943 (2) demonstrated that normal rat and mouse tissues cultivated *in vitro* for prolonged periods underwent a progressive series of changes resulting in the development of some malignant characteristics. This observation of a trend towards malignancy in cell cultures derived from normal tissues has been thoroughly proved by many other investigators, and has become an accepted concept of tissue culture research. Long-term tissue cultures derived from tumors have also been shown to lose their transplantability. This effect was first demonstrated by De Bruyn and Gey (3) in 1952 with mouse lymphoma cells and has subsequently been confirmed by Hsu (4) with rat hepatoma cells, by Foley and Drolet (5) with mouse Sarcoma 180 cells, and by Moore *et al.* (6) with Ehrlich ascites cells of the mouse. Recently, Harris and associates (7) and Watkins and Chen (8) have hybridized Ehrlich ascites cells with mouse fibroblasts or with transformed hamster cells and have shown loss of transplantability associated with some retention of immunoprotective capacity.

In the authors' laboratory, a series of tissue culture lines established (9, 10) from the Ehrlich, TA3, and 6C3HED mouse ascites tumors were found to have lost their transplantability after 3 to 3.5 years of propagation *in vitro*. The establishment of new and still tumorigenic tissue culture lines from the mouse tumors has made it possible to develop a three-component model system for the study of this phenomenon, based on the tumors *in vivo* together with their derived tumorigenic and nontumorigenic cell cultures. The present communication reports the char-

acteristics of this tumor model system, presents data on irradiation effects and on the recovery of transplantability, and shows that the nontumorigenic cells possess a high immunoprotective capacity.

Materials and Methods. Ascites tumors of the Ehrlich, TA3, and 6C3HED lines were carried by weekly serial passage, the Ehrlich and TA3 lines in specific pathogen-free mice of the Connaught strain and the 6C3HED line in C3H mice. Passage was effected by the intraperitoneal inoculation of 2.0×10^6 ascites cells/mouse, a dosage level that invariably caused death within 18 to 21 days. Tissue culture lines of all 3 ascites tumors were established as monolayer cultures in medium 199 (11, 12) supplemented with 20% calf serum, as described previously (9, 10). For routine passage of the cultures, medium 199 supplemented with 5% calf serum was used. Tumorigenicity was determined by injecting intraperitoneally counted numbers of tumor cells (Coulter counter) and allowing 90 days for tumor development. All mice failing to develop ascites tumors within this period were autopsied for possible solid tumor formation. Chromosome analyses of the tumorigenic and nontumorigenic cell cultures were performed by the procedure reported previously for ascites tumors of the mouse (13). Mice were subjected to total body X-irradiation in a Picker therapy machine at 200 kVp and 20 mA.

Recovery of transplantability was studied by injecting groups of mice with nontumorigenic 6C3HED cell cultures suspended in sterile-filtered biological materials. Peritoneal exudate was prepared by injecting mice intraperitoneally with 1.0 ml of incomplete Freund adjuvant (Difco) once a week

for 3 weeks and withdrawing the exudate with a syringe on the fourth week. Mouse serum was prepared from pooled blood obtained by cardiac puncture. Ascites fluid was harvested from 14-day-old tumors with subsequent removal of the tumor cells by centrifugation. All biological materials were passed through UF fritted glass filters to remove any bacteria and tumor cells.

The immunoprotective effect was determined by harvesting nontumorigenic 6C3HED tissue culture cells, washing 3 times with Hanks' balanced salt solution (14) and re-suspending in Hanks' BSS. Groups of mice were injected intraperitoneally with the washed cells, each mouse receiving 5 to 8×10^6 cells once a week for 3 weeks. One week after the final injection, virulent 6C3HED ascites cells, freshly drawn from a mouse tumor, were injected into immunized and normal C3H mice, at different challenge doses. The mice were then observed for tumor development over a 90-day period.

Results. Tumor model. The general characteristics of the tumor model system are presented in Table I. The most striking difference between the tumorigenic and nontumorigenic cell cultures is in their relative transplantability. The tumorigenic cultures regularly produce 100% tumors with 10^2 cells, while the nontumorigenic cultures invariably fail to produce tumors with 10^8 cells. No differences were detected in the relative rate of growth in tissue culture. Some difference was found in the chromosome

TABLE I. General Characteristics of Tumorigenic and Nontumorigenic Ascites Cells.

<i>In Vivo</i>	Grow very slowly in tissue culture 75-78 chromosomes 100% tumors with 10^2 cells
<i>In Vitro</i> , tumorigenic	Grow rapidly in tissue culture Average 117 chromosomes, with 3 or 4 metacentrics 100% tumors with 10^2 cells
nontumorigenic	Grow rapidly in tissue culture Average 123 chromosomes, with 1 metacentric 0 tumors with 10^8 cells

TABLE II. Recovery of Transplantability by 6C3HED Tissue Culture Cells.^a

Mixture injected ^b	Tumor incidence
6C3HED cell control,	
normal C3H mice	0/50
irradiated C3H mice ^c	0/20
6C3HED cells + C3H mouse serum	0/5
+ C3H peritoneal exudate	0/15
+ Swiss peritoneal exudate	0/5
+ 6C3HED ascites fluid	0/20
+ Ehrlich-Lettré ascites fluid	9/19

^a Results averaged from three separate experiments.

^b Each mouse received 1×10^6 viable 6C3HED cells.

^c Mice preirradiated with 350 rads total-body X-irradiation.

complement of the two types of cultures and also between the tissue culture lines and the hypotetraploid state of the *in vivo* tumor lines.

Irradiation studies. To determine whether the nontumorigenic cells in tissue culture had truly lost their transplantability, groups of C3H mice were treated with 350, 550, and 800 rad of total body X-irradiation 24 hr prior to inoculation with 2 to 5×10^6 cells. No ascites tumor formation was observed within 12 to 14 days, at which time the irradiated but uninoculated control mice had begun to die. Accordingly, the surviving inoculated mice were killed and their peritoneal cavities were washed with Hanks' BSS. Microscopic examination of the peritoneal washings showed small numbers of cells resembling ascites tumor cells but inoculation of these cells into normal or X-irradiated C3H mice failed to produce tumors.

Recovery of transplantability. Attempts were made to reinduce transplantability by suspending the nontumorigenic 6C3HED tissue culture cells in various sterile-filtered biological fluids prior to transplantation, with the results presented in Table II. The complete inability of the untreated control cells to produce tumors in either normal or X-irradiated C3H mice is clearly shown. Suspension of the cells in mouse serum or in mouse peritoneal exudate had no effect on

transplantability. Suspension of nontumorigenic 6C3HED cells in sterile ascites fluid derived from the 6C3HED tumor in mice was also ineffective. Surprisingly, however, when nontumorigenic 6C3HED cells were suspended in sterile fluid from the Ehrlich-Létré ascites tumor some recovery of transplantability was achieved, since 50% of the mice in 3 separate experiments developed tumors. These tumors were passaged serially 4 times in normal fashion.

Immunoprotection. Groups of C3H mice, which had received intraperitoneal inoculations of 5 to 8×10^6 nontumorigenic cells once a week for 3 weeks, were challenged with graded numbers of virulent 6C3HED cells with the results shown in Table III. It is evident that the immunized mice were protected completely against challenge with 10^4 , 10^5 , or 10^6 virulent cells while all the nonimmunized control mice died from acites tumors at these challenge doses. Further experiments are in progress to define more completely the high immunoprotective capacity of these nontumorigenic cells.

Discussion. Surprisingly little attention has been paid to the loss of transplantability by tumor cells in long-term tissue culture, although the importance of the phenomenon was pointed out in 1958 (15). Despite current interest (6-8, 16) the mechanism of this loss of transplantability has not yet been established. It appears possible that the cell cultivation procedures may cause the selection of nontumorigenic cells from a heterogeneous population, or that the calf serum used in the culture medium (10) may cause

an alteration in antigenicity leading to rejection by the host animal. The chromosome differences observed between the tumorigenic and nontumorigenic cells suggest that a genetic change may have occurred. Since loss of transplantability in the present study was observed in cell cultures derived from ascites tumors of 3 different cell types, it appears likely that some general mechanism is involved. Whether the observed loss of transplantability represents a regression from the malignant state still remains to be determined.

Earlier studies on this phenomenon (3, 4) had suggested that a gradual decline in transplantability might occur, an observation confirmed recently by Watkins and Chen (8) who found that hybrid Ehrlich cells were capable of producing ascites tumors in immunologically depressed mice but not in normal mice. In the present studies, massive doses of nontumorigenic 6C3HED cells proved unable to elicit ascites tumors when injected into normal or whole-body X-irradiated isogenic C3H mice. Similar studies, employing subcutaneous inoculation, were also completely negative. It would appear that the tissue culture lines employed in the present experiments had completely lost their transplantability.

Attempts were made to reinduce transplantability by suspending the nontumorigenic tissue culture cells in various sterile-filtered biological fluids prior to transplantation. The only positive result obtained was with nontumorigenic 6C3HED cells suspended in fluid from the Ehrlich-Létré ascites tumor. The mechanism of this recovery of transplantability in nonisologous fluid remains to be elucidated. Since the ascites fluid was passed through a UF fritted glass filter before use, any contamination with viable tumor cells must be excluded. Also, the sterile-filtered biological materials themselves proved incapable of eliciting tumor formation. The possibility of a viral relationship or some form of soluble factor, as well as the specificity of the observed phenomenon, are currently under investigation.

A major observation in the present study was the very high degree of immunoprotec-

TABLE III. Immunoprotection against Virulent 6C3HED Ascites Tumor Cells by Nontumorigenic Tissue Culture Cells.

Challenge dose (virulent cells)	Tumor incidence	
	Normal mice	Immunized mice ^a
1.0×10^4	14/14	0/14
1.0×10^5	14/14	0/14
1.0×10^6	14/14	0/14

^a Each mouse received $5-8 \times 10^6$ nontumorigenic cells intraperitoneally once a week for 3 weeks.

tion conferred in mice by the non-tumorigenic tissue culture cells. Complete protection was obtained against challenge with 10^4 , 10^5 , or 10^6 virulent 6C3HED cells, a protection at least tenfold higher than that reported by Watkins and Chen (8) with hybrid Ehrlich cells. On the basis of these results, it would appear that the nontumorigenic tissue culture cells offer a promising new approach to the study of immunoprotection.

Summary. A tumor model system has been developed based on the loss of transplantability by long-term cultures of ascites tumor cells. Nontumorigenic 6C3HED cells were completely unable to produce tumors in both normal and X-irradiated mice. Some recovery of transplantability was achieved by suspending nontumorigenic 6C3HED cells in sterile-filtered ascites fluid from the Ehrlich-Létré tumor. The nontumorigenic 6C3HED cells proved highly immunoprotective in mice since they induced complete protection against challenge with 10^6 virulent cells.

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