MUTANTS OF NONPRODUCER CELL LINES TRANSFORMED BY MURINE SARCOMA VIRUSES*

I. INDUCTION, ISOLATION, PARTICLE PRODUCTION, AND TUMORIGENICITY

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Tumorigenesis in vivo is a important indication of malignant conversion of cells “transformed” in vitro by either chemical, physical, or viral means. A clonal line of nonproducer (NP) cells which was derived from BALB/3T3 cells transformed by the Kirsten strain of murine sarcoma virus (Ki-MSV), is tumorigenic when the cells are inoculated into syngeneic BALB/c mice (1). With this NP line serving as progenitor, we selected morphological variants by cloning after short-term treatment with 5'-bromodeoxyuridine (BrdU). The variants (mutants) were analyzed for tumorigenic potential in vivo and for several markers of viral activity in vitro. The relationship of these markers (cell morphology, chromosome number, production of virion antigens, resusceptibility of sarcoma genome) to in vivo activity has been determined for a number of variants. The results of these studies are presented in this and the following report.

Materials and Methods

Cell lines.—The clonal lines BALB/3T3, clone A31, and the Ki-MSV transformed subclone of this line, K-23 (NP), were obtained from Dr. G. Todaro and S. Aaronson, National Cancer Institute, Bethesda, Md. They were maintained in Eagle's medium (MEM) with 10% fetal bovine serum and antibiotics. The BALB/3T3 cell line is contact inhibited, aneuploid, and nontumorigenic (2). The NP cell line is not contact inhibited, and is highly tumorigenic in immunocompetent BALB/c mice (2). NP cells do not replicate virus, but do contain the murine sarcoma genome which can be rescued by superinfection of the cells with murine leukemia virus (1).

BrdU Treatment.—4 × 10⁵ cells were plated per 50 mm diameter Falcon plastic petri dish (Falcon Plastic, Div. of BioQuest, Oxnard, Calif.). 24 h later the fluid was changed and BrdU (20 μg/ml) was added. The cultures were grown for 24–48 h with BrdU, washed to remove the BrdU; fresh media was then added and the cells further cultivated for several days. The treated cells were trypsinized, and approximately 10² cells seeded per plate in order to isolate possible mutants. Each of the clones isolated initially was recloned by single cell plating several

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1 Abbreviations used in this paper: BrdU, 5'-bromodeoxyuridine; Ki-MSV, Kirsten strain of murine sarcoma virus; MEM, Eagle's medium; NP, nonproducer.

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additional times. Those colonies which were composed mainly of flat cells were chosen for the studies described here.

**Tumorigenicity Test.**—$10^4$–$10^6$ cells in 0.2 ml of MEM were inoculated subcutaneously into 25–35-day old BALB/c mice. The mice were palpated three times per week for 50 days. The progressively growing tumors, which were obtained with several of the cell lines, weighed ½–2 g at this time, while palpable but nonprogressively growing tumors reached less than 0.1 g wet weight. The tumors were also observed histologically.

**Electron Microscopy.**—Shedding of Type C viruses was observed by electron microscopy. Cultures were fixed in situ for 30 min with cold 1% gluteraldehyde, buffered at pH 7.2. Cells were scraped, pelleted by low speed centrifugation, washed thoroughly with phosphate buffer, pH 7.2, fixed for 30 min at 4°C with 1% osmium tetroxide, dehydrated with ethanol, and embedded in epoxy resin (Epon-812). This sections were stained with uranyl acetate and lead citrate and examined in a Hitachi HU-11E electron microscope (Hitachi America Ltd., Indianapolis, Ind.).

**RESULTS**

Each presumptive mutant cell line was tested for two markers; tumorigenesis in syngeneic BALB/c mice, and production of Type C particles. Based on these two properties, the mutants could be classified into four groups: (A) tumorigenic, without particles; (B) tumorigenic, with Type C particles; (C) nontumorigenic, without particles; and (D) nontumorigenic, with Type C particles. Examples of the mutants in each group are shown in Fig. 1.

When the tumorigenic progenitor NP cells were inoculated into BALB/c mice, $10^6$ cells produced a 100% incidence of progressively growing tumors which resulted in death of the host animals within 15–20 days. Clone M-50 in group A, and clone M-57-1 in group B revealed oncogenic potential similar to the NP cells as indicated in Fig. 2. In contrast, the normal progenitor BALB/ 3T3 and clones of groups C and D formed no progressively growing tumors, although some mice in this particular experiment showed tiny palpable tumors after longer incubation periods. These nonprogressive tumors weighed less than 0.2 g. Animals inoculated with $10^4$–$10^6$ cells of C and D group cell lines (10–20 passages after the experiment shown in Fig. 2), did not produce any palpable tumors in the 50 day observation period, as shown in Table I.

![Diagram](https://example.com/diagram.png)

**Fig. 1.** Classification of cell mutant isolates.
Fig. 2. Tumor formation by BrdU-treated NP cells in BALB/c mice. $10^6$ cells in 0.2 ml MEM were inoculated by the subcutaneous route into 35-day old BALB/c mice. Each group contained 6–7 animals. Cells were obtained at 15–20 passages after cloning. Parental NP cells (●—●); M-50 (○—○); M-57-1 (□—□); M-43-2 (▲—▲); M-58-4 (■—■). + denotes all animals sacrificed; ± denotes small nonprogressive growths.

TABLE I

Tumor Formation in BALB/c Mice

| Cell type | Cell line | $10^4$ | Number of cells inoculated per animal* | $10^5$ | $10^6$ |
|-----------|-----------|--------|---------------------------------------|--------|--------|
|           |           | Tumor incidence | Average latency | Tumor incidence | Average latency | Tumor incidence | Average latency |
| Progenitor| NP        | 4/4‡    | 7§          | 4/4    | 10    | 2/4     | 14     |
| A         | M-50      | 4/4     | 10          | 4/4    | 15    | 1/4     | 25     |
| B         | M-57-1    | 4/4     | 8           | 3/3    | 12    | 2/4     | 15     |
| C         | M-43-2    | 0/4     | >50         | 0/4    | >50   | 0/4     | >80    |
| D         | M-58-4    | 0/4     | >50         | 0/4    | >50   | 0/4     | >80    |

* The cells which had 25–30 passages after cloning were inoculated subcutaneously into 25-day old BALB/c mice in 0.2 ml of MEM. The animals were tested for progressively growing tumors three times a week for 50 days. The latency period for tumor development was taken as the time when the tumors first became palpable.

‡ Number of animals with tumors/number of animals inoculated.
§ Days.

Cell mutants of each group were microscopically examined as shown in Fig. 3. The normal progenitor BALB/3T3 appears flat and contact inhibited, while the tumorigenic progenitor NP cell appears highly refractile and round. Tumorigenic potential did not necessarily correspond to morphological flatness, e.g., the M-50 line appears flat despite high tumorigenic potential, while M-57-1.
FIG. 3. Morphology of parental and mutant cells. Magnification 210×. (a) NP parental cell; (b) M-50; (c) M-57-1; (d) BALB/3T3; (e) M-43-2; (f) M-58-4.
of group B, which originally appeared flat was round, refractile, and tumorigenic at the time of these experiments. Both M-43-2 and M-58-4 are flat, and nontumorigenic.

The presence of Type C particles in cloned lines was investigated by electron microscopy. Cell lines in groups B and D are shown shedding typical Type C particles (Fig. 4). Fig. 4 a shows the M-57-1 cell line, Fig. 4 b shows the M-58-4 cell line. Although the cell lines of group A and C did not show particles, tumor explants derived from M-50 cells (group A) occasionally revealed intracytoplasmic A particles as shown in Fig. 4 c. The M-50 cell line, before in vivo transplantation did not reveal intracytoplasmic A particles.

Tumors obtained from groups A and B show typical evidence of malignancy of an epithelial nature with polygonal cells, adenomatous clustering with basement membranes, focal necrosis, many mitotic cells, and minimal leukocyte infiltration (Fig. 5 a, b). Less occasionally, cell mutants in groups C and D produced small but palpable tumors after a long latent period, without progressive growth. The histological observation of these tumors did not indicate malignancy, but rather a granulomatous reaction with enormous leukocyte infiltration. Neither mitotic cells nor necrotic cells were found (Fig. 5 c).

DISCUSSION

The characteristics of each cell mutant produced by BrdU treatment of viral transformed NP cells remained stable for 50 passages after the initial isolation. It is worthwhile to note that the morphological appearance of a mutant line in culture did not necessarily correlate with its tumorigenic potential, since apparent flat variants were sometimes highly tumorigenic, e.g., M-50. Type C particle shedding also did not appear to be correlated with tumorigenesis, e.g., the M-57-1 cell line, while shedding Type C particles, was highly tumorigenic; whereas M-58-4 was not tumorigenic despite abundant shedding of Type C particles. The retention of oncogenic potential in some but not all clones induced by BrdU treatment is contradictory to results recently obtained with mouse melanoma cells treated with BrdU (3). In those experiments (3), short-term or chronic exposure to BrdU resulted in suppression of oncogenicity coincident with a large increase in production of virus particles. However, some variability was noted with time after return to normal medium. In our experiments, the oncogenic properties of the isolated cloned lines remained constant through multiple passages over a 6 mo period. A subsequent paper will focus on the relationship between tumorigenesis and presence of the sarcoma virus genome in these mutant lines.

SUMMARY

A variety of cell mutants were obtained by a single 5′-bromodeoxyuridine (BrdU) treatment of an nonproducer (NP) cell line transformed by the Kirsten strain of murine sarcoma virus (Ki-MSV). Isolation procedures of these cell
FIG. 4. Electron microscopy of mutant cell lines: (a) M-57-1 cells showing Type C particles, 15,000X; (b) M-58-4 cells showing Type C particles, 22,500X; (c) M-50T cells showing intracisternal A particles, 22,500X.
Fig. 5. Morphological appearance of tumors derived from mutant cell lines. Cells stained with Giemsa. Magnification 77× (left-hand panel); 187× (right-hand panel). Top (a, d) M-50 epithelial tumor; middle (b, e) M-57-1, carcinomatous appearance; bottom (c, f) M-58-4, granulomatous appearance.
mutants are described. The cell mutants obtained were classified by tumorigenic potential and shedding of Type C virus particles. The cell mutants were classified into four groups: (A) tumorigenic, without particles; (B) tumorigenic, with Type C particles; (C) nontumorigenic, without particles; and (D) nontumorigenic, with Type C particles. The tumorigenic cell lines showed variability in morphology with both flat and typical transformed appearing cell lines showing equal transplantability.

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