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Sarcomas Routinely Produced From Putatively Nontumorigenic Balb/3T3 and C3H/10T1/2 Cells by Subcutaneous Inoculation Attached to Plastic Platelets

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The Balb/3T3 and C3H/10T1/2 lines, noted for their marked postconfluence inhibition of proliferation and anchorage dependence, and frequently studied as nontumorigenic lines that are compared with tumorigenic sublines transformed with various agents, produced tumors within two to four months at low-cell dosage (3×10^4 cells) when implanted subcutaneously attached to $1 \times 5 \times 10$ mm polycarbonate platelets. Platelets alone did not produce tumors. The cultured Balb/3T3 tumor cells showed loss of both postconfluence inhibition of proliferation and anchorage dependence. Tumors arising from attached Balb/3T3 cells in (BALB/c × C57B1/6)F1 hybrids were shown to be transplantable to BALB/c but not to C57B1/6 mice, proving that the tumors were derived from Balb/3T3 and not from host cells. The tumors exhibited unique transplantation rejection antigens that did not cross-react with each other. Scanning electronmicroscopy of Balb/3T3 cells and derived tumor cells on Teflon® substrates (on which only the tumor cells and not the parent Balb/3T3 cells could grow) revealed that the two cell types were remarkably similar in appearance, except that the tumor cells were larger and showed many more microvilli that tended to concentrate over the nucleus. We conclude that Balb/3T3 cells and C3H/10T1/2 cells are preneoplastic and give rise to spontaneously transformed clones when implanted in vivo attached to a solid substrate.

Key words: actin filament bundles, LETS protein, cytoskeleton, chick embryo fibroblasts, triton cytoskeleton, nonmuscle actin

INTRODUCTION

The Balb/3T3 line (1), and more recently the C3H/10T1/2 lines (28), have been used by many investigators as prototype nontumorigenic lines suitable for morphological and functional comparison with tumorigenic sublines derived from them by in vitro transformation with viruses (1, 2), by chemicals (3), and by manipulations that encourage the appearance of spontaneous transformation (4, 5). The various properties of Balb/3T3 cells most recently compared with those of transformed sublines are [see (6) for earlier references]: postconfluence inhibition of proliferation (7, 8), serum growth factor requirements (9), and response to other growth factors (10, 11), revertants of SV40 and MSV-transformed lines (12, 9, 13), lectin-binding and agglutination (14), membrane transport phenomena (15–18), surface biochemistry (19–21), and alteration of surface antigens (5).

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If Balb/3T3 or C3H/10T1/2 cells are implanted subcutaneously in relatively low doses (3×10^4 cells) attached to $1 \times 5 \times 10$ mm polycarbonate plastic platelets, sarcomas arise in the majority of cases in two to four months. Both cell lines exhibit marked anchorage dependence, or the inability to divide in vitro unless attached to a solid substrate. This property is assayed by the inability of anchorage-dependent cells to grow suspended in soft agar or, more recently, by their inability to divide on a transparent film of Teflon (23). The implantation of Balb/3T3 and C3H/10T1/2 cells attached to a solid substrate appears to satisfy their immediate requirement for anchorage dependence in vivo during an initial period prior to tumor development. This article contains a brief summary of recent studies on vasoformative sarcomas produced by the subcutaneous implantation of substrate-attached Balb/3T3 cells published in detail elsewhere (24), plus preliminary findings on the production of sarcomas from plastic substrate-attached C3H/10T1/2 cells and on the appearance by scanning electronmicroscopy of Balb/3T3 cells and derived tumor cells on Teflon, a nonadhesive substrate on which only the tumor cells grow.

METHODS

Cell Lines

Clone A31 Balb/3T3 cells were obtained from the American Type Culture Collection, Rockville, Maryland. Clone 8 C3H/10T1/2 cells were obtained from Charles Heidelberger, M.D., McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, Wisconsin. The Balb/3T3 cells were maintained in the Dulbecco-Vogt modification of Eagle's MEM containing 10% fetal bovine serum. The C3H/10T1/2 cells were maintained in Eagle's BME containing 10% fetal bovine serum. All media contained penicillin, 100 units per ml, and streptomycin, 100 micrograms per ml. The cells were kept in continuous exponential growth phase in a CO_2 incubator for less than three weeks from the time of receipt until their use in the experiments described.

Subcutaneous Inoculation of Cells Attached to Plastic Platelets

Batches of polycarbonate platelets measuring $1 \times 5 \times 10$ mm were cut from 1-mm thick sheets of polycarbonate plastic, left in 1 liter of 0.5% of Calgolac® with continuous stirring for 24 hr at room temperature, and thoroughly rinsed in running tap water followed by repeated changes of distilled water. The platelets were sterilized by autoclaving and placed in medium to which enough cells were added to form a confluent monolayer 24 hr later. The number of confluent cells per platelet averaged 3×10^4. Platelets with attached Balb/3T3 cells were implanted between the dermis and panniculus carnosus (24); those with attached C3H/10T1/2 cells were simply inserted subcutaneously, with the cells facing the epidermis.

Scanning Electron Microscopy

Cells were planted at low density on Teflon substrates as previously described. Then, 20 and 48 hr later, the attached cells were washed twice with 0.1 M phosphate buffer (PB), pH 7.4, fixed in 2% glutaraldehyde for 30 min, washed twice with PB, and postfixed for 15 min in 1% OsO_4 in PB. All procedures were carried out at 37°C. The attached cells were rapidly dehydrated in an ascending alcohol series, followed by an ascending series of amyl acetate in alcohol to 100% amyl acetate, and critical point was

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dried according to the method of Anderson (25). The cells were then mounted on specimen stubs, coated with carbon followed by approximately 150 angstroms of gold, and examined in an Etec Autoscan Microscope.

Immunization and Testing for Transplantation Rejection (TR) Antigens Arising From Plastic Platelet Attached Balb/3T3 Cells

Pieces of tumor measuring 1 × 1 × 3 mm were implanted subcutaneously by trocar in adult male BALB/c mice. Fifteen to 25 days later the tumors were ligated tightly around their base with a rubber band. The ligature was released 24 hr later, following which the tumor regressed within 5–7 days. These animals then received two subsequent bimonthly booster subcutaneous inoculations with trocar pieces of tumor, and were used within three weeks. To test for the presence of immunity against a given tumor, immunized animals were challenged with 1 × 1 × 3 mm pieces of the tumor and observed for two months or until they died of tumor.

RESULTS AND DISCUSSION

Gross and Microscopic Characteristics of Tumors Arising From Balb/3T3 and C3H/10T1/2 Cells Attached to Plastic Platelets

Tumors arose in 6 of 8 mice implanted with plastic platelet attached Balb/3T3 cells within 8 to 12 weeks, and in 10 of 27 mice implanted with plastic platelet attached C3H/10T1/2 cells within 14 weeks. No tumors were seen in BALB/c mice implanted with platelets alone after one year, nor in C3H mice after eight months. Grossly, the plastic platelets were found either immediately below the tumor capsule or in some cases imbedded in the tumor. The Balb/3T3 tumors were soft and pink on cross section, whereas the C3H/10T1/2 tumors were firm, pale, and translucent. Microscopically, the Balb/3T3 tumors showed many blood vessels lined by tumor cells and were given a diagnosis of "vasoformative sarcoma" (Fig. 1C). The C3H/10T1/2 tumors consisted of a large number of multinucleated giant cells interspersed among plump parallel-oriented spindle cells and were given a diagnosis of fibrosarcoma (Fig. 1D). The tissue of origin of the Balb/3T3 cells appeared to be most consistent with vascular endothelium; that of the 10T1/2 cells appeared most likely to be fibroblastic tissue.

Proof That the Tumors Arising From Plastic Attached Balb/3T3 Cells Did Not Arise From Host Tissue

Tumors arising in (BALB/c × C57BL/6) F1 hybrids implanted with substrate attached Balb/3T3 cells were found to be transplantable to BALB/c mice but not to C57BL/6 mice. Had the tumors been derived from host tissue, they would not have grown in either parent strain.

Loss of Postconfluence Inhibition of Proliferation and Anchorage Dependence by the Balb/3T3 Tumor Cells

A tissue culture line was established from one of the vasoformative sarcomas arising within eight weeks from Balb/3T3 cells inoculated and subcutaneously attached to 3-mm glass beads (6). The saturation density of growth curves of the tumor cells, designated HB4 cells, was 16×10^4 per square centimeter compared with a saturation density of 6.3×10^4 per square centimeter for the original A31 clone of Balb/3T3 cells grown under identical conditions. The HB4 cells also exhibited loss of anchorage dependence in vivo

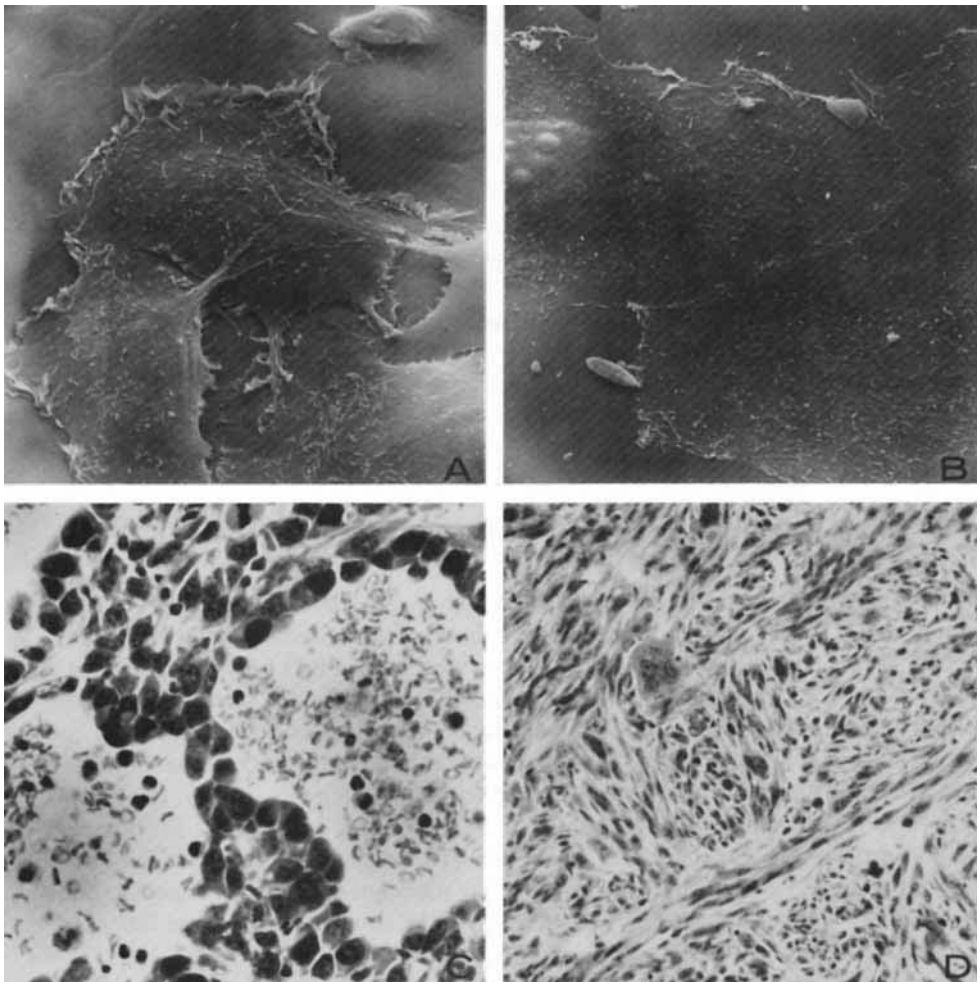

Fig. 1. (A) Balb/3T3 cells on Teflon. × 1,400. (B) HB4 tumor cells (derived from substrate-attached Balb/3T3 cells) on Teflon. × 700. (C) Histologic section of Balb/3T3 tumor showing vascular channels formed by tumor cells. Approximately × 280. (D) Histologic section of C3H/10T1/2 tumor. Approximately × 230.

and in vitro. In vivo, they no longer required attachment to a solid substrate in order to grow as a tumor: a subcutaneous inoculation of 10^5 cells in suspension produced tumors in seven of ten mice. In vitro, the HB4 cells proliferated rapidly on Teflon, a nonadhesive substrate, whereas the original Balb/3T3 cells did not. Ability to proliferate on Teflon is a useful measure of loss of anchorage dependence analogous to growth in soft agar or methylcellulose (23).

Tumor-unique Transplantation Rejection (TR) Antigens of the Balb/3T3 Tumors

Table I shows that tumors arising from substrate attached Balb/3T3 cells in different animals possessed transplantation-rejection antigens that were unique for each individual tumor. Two important conclusions may be drawn from these facts: (1) The tumors were of clonal origin, that is, they arose from a single cell in the substrate attached monolayer

TABLE I. Tumor-unique, Noncrossreacting, Tumor-rejecting Antigens in Four Tumors Arising in Different Animals From Implants of Plastic-attached Balb/3T3 Cells

Tumors used for immunization	No. died with tumor / no. challenged with trocar doses of:			
	P3/1	P3/2	P3/3	P3/4
P3/1	1/10	10/10	10/10	N.D.*
P3/2	10/10	0/14	9/10	10/10
P3/3	10/10	10/10	1/11	10/10
P3/4	N.D.	10/10	10/10	2/11
None	10/10	10/10	9/10	10/10

*N.D. = not done

in vitro and (2) there were no tumorigenic cells already present in the substrate attached Balb/3T3 monolayers at the time of implantation, because if there had been they would have overgrown the confluence-inhibited monolayer and produced tumors in different animals that cross-reacted antigenically.

Scanning Electronmicroscopy of Balb/3T3 and Derived Tumor Cells (Designated HB4 Cells) on Teflon Substrate

In spite of the fact that Balb/3T3 cells do not grow on Teflon (23), they remained flat and stretched out (Fig. 1A) with no outstanding morphological differences compared with their appearance on glass (not shown). The HB4 cells appeared larger and exhibited many more microvilli that tended to concentrate over the nucleus. In general, the HB4 cells appeared somewhat flatter, and frequently formed nonoverlapping cell-to-cell contacts similar to those exhibited by the Balb/3T3 cells (Fig. 1B). Even though the HB4 cells were tumorigenic when inoculated in suspension at a dose of 10^5 cells, they did not show the marked criss-cross pattern characteristic of Balb/3T3 cells transformed in vitro by SV40 virus.

Balb/3T3 Cells: Preneoplastic or Neoplastic?

Balb/3T3 cells cannot be considered frankly neoplastic, because if they were the probability is high that they would have produced tumors with cross-reacting rejection antigens. The evidence favors characterization of Balb/3T3 cells as preneoplastic, that is, altered in the direction of neoplastic transformation by loss or gain of an unknown number of characters, but still retaining the property of solid substrate anchorage dependence which prevents them from forming a tumor when inoculated in vivo in suspension. The appearance of spontaneous transformants which have lost anchorage dependence in vivo is closely analogous to the transformants that are occasionally seen in vitro in confluent monolayers of Balb/3T3 cells (22) and in C3H/10T1/2 cells maintained in Eagle's Minimum Essential Medium instead of the usual Eagle's Basal Medium and in the confluent state for 3–4 weeks without medium change (26). It is probably the overgrowth of these confluence-insensitive spontaneous transformants that accounts for the known ease with which a culture of Balb/3T3 cells becomes tumorigenic if carried as a confluent culture for any length of time (27).

Loss of anchorage dependence appears to be a highly significant mutation (in the sense of a permanent heritable alteration of phenotype) in the sequence of mutation-selectional steps affecting cellular character that permits progression toward increasingly successful malignant behavior.

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