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Different calcium requirements for proliferation of conditionally and unconditionally tumorigenic mouse cells

[DNA synthesis/neoplastic transformation/(BALB/3T3 cells)/(C3H/10T1/2 cells)]

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ABSTRACT Conditionally tumorigenic BALB/3T3 mouse cells (which produce tumors in BALB/c mice only under special conditions) cannot sustain DNA synthesis and consequently stop proliferating in media containing low concentrations (0–0.02 mM) of physiologically available calcium. By contrast, cells that have been neoplastically transformed (tumorigenic in mice without special assistance) *in vitro* by different oncogens, can sustain DNA synthesis and proliferate in such calcium-deficient media. The possible importance for tumor growth of an ability to withstand calcium deprivation is examined. It is suggested that this property may prove to be a reliable indicator of neoplastic transformation.

The availability of calcium ions limits the initiation of DNA synthesis and proliferation of a variety of cells, ranging from *Streptococcus lactis* and *Physarum polycephalum* to BALB/3T3 mouse cells *in vitro* and rat hepatocytes *in vivo* (1–23). The possibility that a change of calcium's regulatory function is involved in neoplastic transformation has been raised by Balk and his associates (1, 2), because chicken fibroblasts infected with the oncogenic Rous sarcoma virus proliferate rapidly in the presence of an extracellular ionic calcium concentration that is too low to allow the proliferation of uninfected fibroblasts. This suggestion is supported by the observation of Swierenga *et al.* (15, 17) that cells from several rat (nickel sulfide-induced) rhabdomyosarcomas, but not cells from the corresponding non-tumorous muscle tissues, proliferate indefinitely in an ionic calcium-deficient medium. In the present communication, we will also support this proposal by showing that several variously transformed (i.e., unconditionally tumorigenic in BALB/c mice) derivatives of BALB/3T3 mouse cells withstand an ionic calcium deficiency that proliferatively paralyzes cells of the conditionally tumorigenic (i.e., tumorigenic only under special conditions; 24) parental BALB/3T3 line.

MATERIALS AND METHODS

BALB/3T3 mouse fetal cells (clone A-31, passage 87) were obtained from the American Type Culture Collection (Rockville, Md.). These conditionally tumorigenic cells probably originated from vascular endothelial cells because they produce hemangioendotheliomas when introduced into BALB/c mice on glass beads (24). Parenthetically, an endothelial origin of these cells is consistent with our observation that all of the unconditionally tumorigenic (i.e., neoplastically transformed) lines produced malignant tumors resembling hemangioendotheliomas.

Several strains of unconditionally tumorigenic derivatives of BALB/3T3 cells (25, 26), which, unlike BALB/3T3 cells, form tumors in BALB/c mice without prior attachment to glass beads, were gifts from Dr. G. Todaro of the National Cancer Institute (Bethesda, Md.). These derivatives were: K-BALB/3T3, transformed *in vitro* by the Kirsten (RNA-containing) murine sarcoma virus (26); MC5-5 BALB/3T3 transformed *in vitro* by methylcholanthrene; SV-BALB/3T3 (T), transformed *in vitro* by the (DNA-containing) simian virus 40. A second culture of simian virus 40 transformed BALB/3T3 cells, SV-BALB/3T3 (F) was obtained from Flow Laboratories (Rockville, Md.). Highly tumorigenic BALB/3T12 cells (selected by passage of BALB/c mouse fetal cells at high densities; 25) were obtained from the American Type Culture Collection. Finally, MCA (Type III)-C3H/10T1/2 (clone 15, passage 8), a highly tumorigenic (malignant fibrosarcoma) line of C3H mouse fibroblasts transformed *in vitro* by methylcholanthrene (27), was a gift from Dr. C. H. Heidelberger of the McArdle Laboratory for Cancer Research (Madison, Wisc.) Before undertaking these experiments we confirmed the (unconditional) tumorigenicity of all of these lines.

BALB/3T3 cells and their (unconditionally) tumorigenic derivatives were cultivated in a medium consisting of 90% (vol/vol) Dulbecco's modified Eagle's medium (DEM; Grand Island Biological Co., Grand Island, N.Y.), 10% (vol/vol) bovine calf serum (Colorado Serum Co., Denver, Colo.), and 50 μg/ml of gentamicin (Microbiological Associates Inc., Bethesda, Md.). Unconditionally tumorigenic mouse fibroblasts of the MCA (Type III)-C3H/10T1/2 line were maintained in an antibiotic-free medium consisting of 90% (vol/vol) Eagle's basal medium with Hanks' salts (BME; Grand Island Biological Co.) and 10% (vol/vol) heat-inactivated fetal bovine serum (Colorado Serum Co.).

Low-calcium serum-DEM medium was prepared by mixing 90% (vol/vol) calcium-free DEM with either 10% (vol/vol) bovine calf serum or adult rat "plasma", the ionic (physiologically available) calcium contents of which were reduced to 0.01 mM with [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA) according to procedures previously described (3–5, 28). Low-calcium serum-BME medium was prepared by separately reducing the calcium levels of both BME and fetal bovine serum to the desired levels with EGTA. EGTA is a specific calcium chelator which would not affect the concentrations of other divalent cations such as Mg^{2+}. Moreover, Ca-EGTA does not by itself affect DNA synthesis or cell proliferation (1–5, 19).

The rat "plasma" used in some experiments was prepared from the blood of adult (250–300 g), male, specific-pathogen-free albino rats that had been bred in this laboratory. Blood was removed from the dorsal aorta of each anesthetized (with Fluothane®; Ayerst Laboratories, Montreal, Canada) rat with a cooled, 18-gauge needle (fitted with Tygon tubing), and, apart from the first several drops, was collected in cooled (0°C), siliconized Vacutainer® tubes (Becton-Dickinson, Rutherford, N.J.). Platelets and blood cells were sedimented at 0° by centrifugation (1000 × g for 30 min), and the supernatant plasma

Abbreviations: EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; DEM, Dulbecco's modified Eagle's medium; BME, Eagle's basal medium with Hanks' salts.

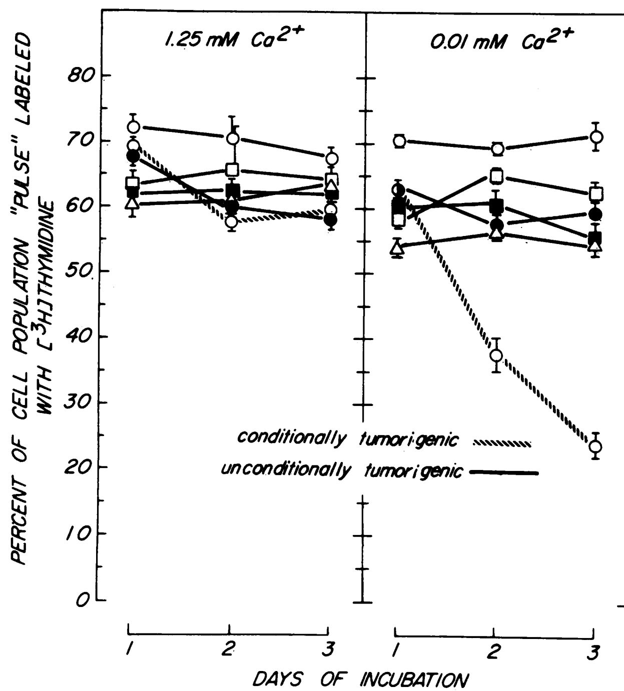

FIG. 1. The ability of *unconditionally* tumorigenic K-BALB/3T3 (O—O), MC5-5-BALB/3T3 (●—●), SV-BALB/3T3 (T) (■—■), SV-BALB/3T3 (F) (□—□), and BALB/3T12 (△—△) mouse fetal cells to maintain their DNA-synthetic activity in low-calcium serum-DEM medium, which does not support prolonged DNA-synthesis by *conditionally* tumorigenic BALB/3T3 mouse fetal cells (O-O). On day 0, all cells were seeded on coverslips at a density of 0.4×10^4/cm^2 in medium consisting of 90% (vol/vol) calcium-free DEM and 10% (vol/vol) bovine calf serum, the physiologically available calcium concentration of which was reduced to 0.01 mM with EGTA. The physiologically available calcium concentration in half of the cultures (the controls) was immediately raised to 1.25 mM by the addition of $CaCl_2$. Thus, all cultures contained the same concentration of Ca-EGTA. The points are the means ± SEM of values from four cultures.

was heated at 56° for 30 min, then filtered, frozen, and stored at −20°. Although this "plasma" contained no anticoagulants, it did not clot during isolation or subsequent use, probably because of heat inactivation of factors V and VII.

The proportions of DNA-synthesizing cells in cultures grown on coverslips were determined by exposing the cultures for 1 hr to 10 μCi/ml of [^{3}H]thymidine (specific activity, 20 Ci/mmol; New England Nuclear Corp., Boston). The coverslips were then rinsed twice with phosphate-buffered saline (pH 7.2), fixed in a solution consisting of nine parts of phosphate-buffered neutral formalin and one part of glacial acetic acid, and finally washed twice with a 10 mM solution of nonradioactive thymidine. The cells were covered with a layer of Kodak NTB-2 nuclear track emulsion (Eastman Kodak, Rochester, N.Y.). Two days later background-free autoradiographs were prepared and the cells were stained according to the procedures of Whitfield *et al.* (19, 22).

The cell densities in cultures seeded in petri dishes (as opposed to coverslips) were determined by suspending the cells in 2.0 ml of a 0.25% solution of trypsin in phosphate-buffered saline, diluting the suspension with Isoton cell counting solution (Coulter Electronics, Hialeah, Fla.), and determining the cell concentration with a model F Coulter electronic cell counter.

RESULTS

During 3 days of incubation of a population of conditionally tumorigenic BALB/3T3 cells in their normal, growth-pro-

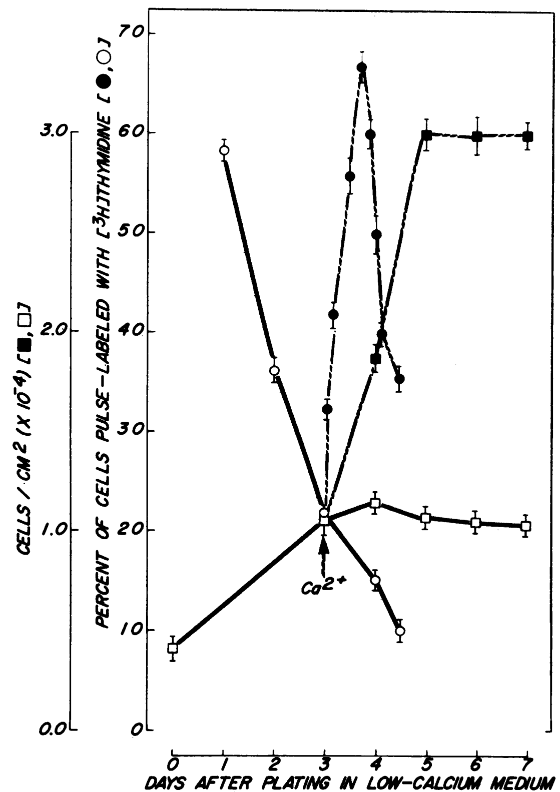

FIG. 2. A demonstration of the reversible blockage by calcium-deprivation of the *conditionally* tumorigenic BALB/3T3 cell's growth-division cycle at the G1/S boundary. Cells were seeded (at a density of 0.4×10^4 per cm^2 of petri dish surface) at time "0" in 2.0 ml of 90% DEM–10% bovine calf serum containing 0.02 mM calcium, which was replaced 24 hr later with fresh, low-calcium medium. After a further 48 hr (or a total of 72 hr) of calcium deprivation, the extracellular (physiologically available) calcium concentration was either left at 0.02 mM (O,□) or adjusted to 1.25 mM by adding 125 μl of a 20 mM $CaCl_2$ solution to the medium (●,■). The proportion of cells making DNA (i.e., which were pulse-labeled with [^{3}H]thymidine) was then determined autoradiographically, and the cell number per cm^2 was determined with a Coulter model F electronic cell counter. The points are means ± SEM of the values from four to eight cultures.

moting serum-DEM medium (containing 1.25 mM nonchelated calcium), there was a constant flow of cells through the S phase of the cell cycle, and the proportion of DNA-synthesizing cells accordingly remained between 60 and 70% (Fig. 1). As would be expected from previous observations (3–5), the proportions of DNA-synthesizing cells steadily declined and proliferation eventually stopped during 3–4.5 days of incubation of BALB/3T3 cultures in serum-DEM medium containing 0.01 mM or 0.02 mM physiologically available calcium (Figs. 1 and 2). Calcium deprivation caused most of the cells in these conditionally tumorigenic cultures to collect at the G1/S boundary, because returning the extracellular, physiologically available calcium concentration to 1.25 mM caused the large group of blocked cells almost immediately to initiate DNA synthesis and start dividing several hours later (Fig. 2).

During the same period of incubation in "high"-calcium medium, the proportion of DNA-synthesizing cells in cultures of the unconditionally tumorigenic cell lines also remained between 60 and 75 percent (Fig. 1). However, in striking contrast to the conditionally tumorigenic BALB/3T3 cells, the unconditionally tumorigenic lines maintained high levels of DNA-synthetic activity for at least 3 days after the physiologically available calcium level was lowered from 1.25 mM to 0.01

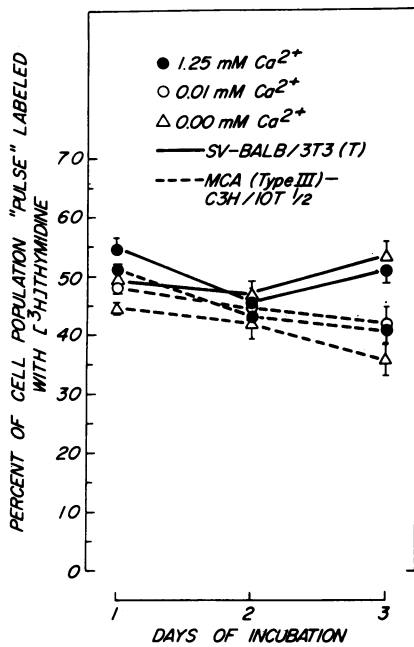

FIG. 3. Two examples of the ability of *unconditionally* tumorigenic cells to maintain DNA-synthetic activity even in the total absence of physiologically available calcium. On day 0, all cells were seeded at a density of 0.4×10^4/cm^2 on coverslips in low-calcium (0.01 mM) medium consisting of 90% (vol/vol) calcium-free DEM plus 10% (vol/vol) EGTA-treated bovine calf serum [SV-BALB/3T3 (T)] or 90% (vol/vol) BME plus 10% (vol/vol) heat-inactivated fetal bovine serum [MCA(Type III)-C3H/10T1/2]. The ionic calcium concentration was either left at 0.01 mM, or further reduced to 0.00 mM by addition of 5 μl of a 5.0 mM solution of EGTA. In the remaining cultures the ionic calcium level was raised to 1.25 mM by addition of $CaCl_2$. The points are the means ± SEM of the values from four cultures.

mM (Fig. 1). Moreover, unconditionally tumorigenic cells [including the differently derived MCA (Type III)-C3H/10T1/2 fibroblasts] maintained a high level of DNA-synthetic activity even in serum DEM (or BME) medium containing no physiologically available calcium (Fig. 3).

Finally, unconditionally tumorigenic cells also effectively withstood severe calcium deprivation in a more "physiological" medium consisting of 90% (vol/vol) DEM and 10% (vol/vol) of the phylogenetically closer adult rat "plasma", the components of which (unlike those of bovine sera) had not been modified by the clotting process. For example, the proportion of DNA-synthesizing cells rapidly dropped in BALB/3T3 cultures in very low (0.002 mM)-calcium "plasma" medium, but the proportion actually *increased* in corresponding cultures of the very highly tumorigenic K-BALB/3T3 line (Fig. 4).

DISCUSSION

BALB/3T3 (clone A-31) cells may be considered as conditionally transformed because they produce tumors only when introduced into BALB/c mice on glass beads (24). In this conditionally tumorigenic state, the cells cannot maintain DNA synthetic activity and proliferate in an ionic calcium-deficient medium. Upon neoplastic transformation (by various oncogens), prior anchorage to glass beads is no longer needed for tumorigenesis and the cells can withstand severe calcium deprivation. Since the proliferative machineries of cells from rat rhabdomyosarcomas (15, 17) and Rous sarcoma virus-infected chicken fibroblasts (1, 2) also withstand severe ionic calcium deprivation (which stops proliferation of their normal equiv-

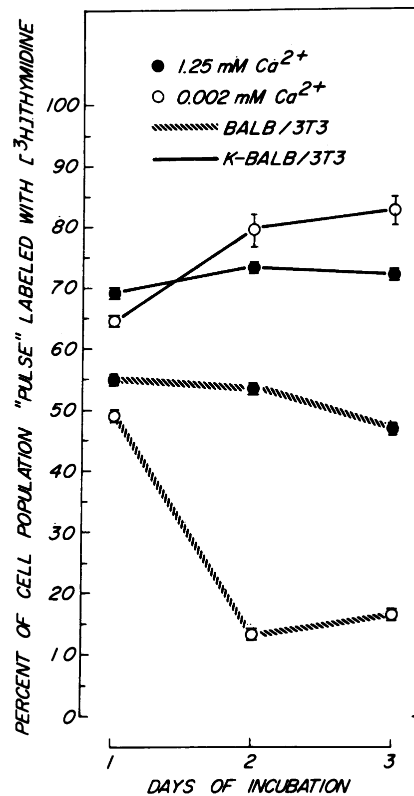

FIG. 4. The ability of *unconditionally* tumorigenic K-BALB/3T3 cells, but not *conditionally* tumorigenic BALB/3T3 cells, to maintain a very high DNA-synthetic activity in a low-calcium adult rat "plasma"-DEM medium. On day 0, all cells were seeded on coverslips at a density of 0.4×10^4 per cm^2 in 90% (vol/vol) calcium-free DEM plus 10% (vol/vol) adult rat "plasma", the physiologically available calcium concentration of which had been reduced to 0.002 mM with EGTA. The ionic (physiologically available) calcium concentration in half of the cultures was immediately raised from 0.002 to 1.25 mM by the addition of $CaCl_2$. It should be noted that 10^6 K-BALB/3T3 cells invariably produced large tumors resembling hemangioendotheliomas within only 8 days after subcutaneous injection into normal BALB/c mice. The points are the means ± SEM of values from four cultures.

alents), an elimination of calcium's regulatory function may be part, or at least a side-effect, of the neoplastic transformation of cells of mesenchymal origin.

The available evidence (20) points to calcium ions being introduced into the prereplicative (G1) reaction sequence by the brief elevation of the cellular cyclic AMP content that precedes, and participates in, the initiation of DNA synthesis by several types of cell (29–37; A. L. Boynton and J. F. Whitfield, unpublished observations), including BALB/3T3 cells (31). This brief elevation of the cyclic AMP level probably mobilizes mitochondrially-sequestered calcium ions (38–40), which may then stimulate some DNA-synthetic enzyme [such as thymidylate synthetase (41)] either directly, or indirectly [as in *Streptococcus lactis* (10)] by inactivating a repressor. Alternatively, these ions might cause a timely release of such enzyme(s) from inactive complexes in the same way they release neurotransmitters from secretory granules and synaptic vesicles (42).

The proliferative machineries of neoplastic cells could withstand calcium deprivation for a variety of reasons. In one case, the calcium-cyclic AMP system might become functionless through loss of the mitochondrial calcium-sequestering capacity, which would stabilize the cytosol ionic calcium con-

centration at an elevated, stimulatory level. In other cases, the DNA-synthetic process might be uncoupled from its calcium-cyclic AMP controls by the cell's failure to synthesize a calcium-sensitive repressor, or to sequester DNA-synthetic enzyme(s) in a calcium-mobilizable form.

It is not known whether proliferative insensitivity to ionic calcium deprivation is necessary for, or merely signals the completion of, neoplastic transformation. However, the anchorage dependence of tumorigenesis by conditionally tumorigenic BALB/3T3 cells could be due to the ability of the calcium-cyclic AMP mechanism to operate only in cells attached to a suitable surface. Such a coupling of the mobilization of intracellular calcium ions to anchorage to a suitable surface would account for the restriction of the proliferation of normal cells *in vivo* to certain regions of a tissue such as the basement membranes (43, 44). Removal of the calcium-cyclic AMP controls from the DNA-synthetic machinery would eliminate the requirement for attachment to a specific substratum, and thereby endow the cell with a potential for unlimited and widespread proliferation.

Finally, despite the obvious uncertainties of its mechanism and role in tumorigenesis, the property of being able to proliferate in an ionic calcium-deficient, EGTA-containing medium may eventually prove to be a reliable indicator of neoplastic transformation.

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