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Ornithine Decarboxylase Activity in Cells Acutely and Chronically Transformed by Murine Sarcoma Virus (40301)

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Polyamine biosynthesis is one of the earliest events occurring during cellular proliferation (1). Ornithine decarboxylase (ODC), which catalyzes the formation of putrescine from ornithine, is the rate limiting enzyme in polyamine biosynthesis (2). Resting cells have low, stable ODC levels which increase rapidly upon the onset of growth (3, 4). We have demonstrated that increased ODC activity follows infection of cultured mouse Balb/3T3 (B/3T3) cells with murine sarcoma virus (MSV) (5). The increase in ODC activity is independent of the population doubling time and commences immediately prior to morphological transformation. Elevation of ODC levels also precedes morphological transformation by Rous sarcoma virus (6).

Transforming stocks of MSV consist of mixtures of defective transforming virus and non-transforming murine leukemia virus (MuLV) (7). The MuLV is usually present in great excess, and dual infection of mouse cells with both viruses is required for MSV replication. Cells infected with the transforming virus alone undergo transformation, and retain the sarcoma genome, but do not release infectious virus. Two such classes of transformed cells have been described: (a) Non-producer (np) which do not release virus particles (8), and (b) sarcoma virus positive, leukemia virus negative (S+L−) cells which release noninfectious virus particles and have MuLV gs antigen (8). Superinfection of both of these transformed cell classes with MuLV results in release of infectious transforming and nontransforming viruses. However, superinfected S+L− cells undergo further morphological alteration (thus providing a focus assay for MuLV). Superinfection of np cells does not result in morphological alteration.

In this communication we describe experiments studying the relationship between elevated ODC activity, virus induced morphological transformation, virus production, and rates of cellular division. We compare producer, np and S+L− derivatives of a single murine cell clone.

Materials and methods. Cell lines. B/3T3, clone A31, is a contact inhibited, 'flat' non-virus releasing cell (9). It becomes transformed after MSV infection, but productive infection with MuLV does not induce morphological change. D245E6 is a S+L− B/3T3 clone selected for its relative 'flatness'. After MuLV superinfection, its morphology becomes more transformed (10). KA31 is a Kirsten MSV transformed np clone of B/3T3 (11). MuLV superinfection of KA31 results in release of transforming and nontransforming viruses without morphological change. B/3T3 and KA31 cells were obtained from Dr. Stuart Aaronson and D245E6 cells from Dr. Robert Bassin. Cells were maintained in 75 mm flasks in 5% CO_2 atmosphere at 37°. Fluids were changed at 24 or 48 hr intervals. Cells were grown in Eagle's essential medium (D245E6) or Dulbecco's modification of it (B/3T3 and KA31). Medium was supplemented with 10% heat inactivated (56°, 30 min) fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 μg/ml).

Viruses and virus assays. Gz-MSV, a mixture of transforming and nontransforming viruses, was recovered from the supernatant fluids of acutely infected B/3T3 cells, and had a titer of 2×10^6 focus forming units/ml (12). Rauscher leukemia virus, a strain of MuLV, was obtained by concentrating the supernatant fluids of chronically infected BALB/c JLSV-9 cells, and had a titer of 1×10^7 plaque forming units/ml. Infectious center assays were modifications of the commonly used methods for assays of MSV and MuLV (8, 13). Fifty or 100 mitomycin C

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treated (25 μg, 1 hr) test cells were added onto sparsely seeded indicator cells. For MSV assays, the indicator cells were B/3T3, and foci of refractile MSV transformed cells were enumerated 5 days later. For MuLV assays, S+L− Al-2 cells (14) were used as indicator cells, and plaques (consisting of supertransformed cells that had lysed or floated away) enumerated 5 days later. Colony forming efficiency (CFE) in semi-solid medium was determined by suspending 1×10^3 viable cells in 0.4% agarose over a 0.9% agarose base. After 1, 8 and 15 days, another layer of agarose was placed over the cell containing layer. Colonies were counted 18 days after seeding.

Other Methods. ODC activity was assayed by measuring enzyme released CO_2 as described previously (5). Replicate plates were rinsed twice with saline and frozen (−20°) until assayed. Cells were gently scraped into buffer, freeze-thawed three times, and centrifuged (4500*g* for 10 min). Supernatant fluids (0.25 ml) were incubated with 50 μl [^{14}C]-ornithine in plastic tubes equipped with a rubber stopper supporting a polyethylene center well. After incubation (37°, 45 min), 0.2 ml hydroxide of hyamine was added to each well. After a further incubation of 15 min, 0.2 ml of perchloric acid was added to each well. Tubes were agitated for 15 min to release bound CO_2, the center wells were removed, and their radioactivities determined. Protein was determined by the Lowry method (12). Cells were counted with a hemocytometer, and viability determined by trypan blue exclusion.

Results. Properties of the cell lines used are presented in Table I. Uninfected B/3T3 cells were epithelioid and contact inhibited, did not release virus, and failed to grow in soft agarose. Productive infection with MuLV did not alter its morphology. Within 48 hr of MSV infection, B/3T3 cells became round or spindle shaped, were highly refractile and adhered poorly to the substrate. Morphological transformation was accompanied by release of transforming and nontransforming viruses, and the ability to grow in soft agarose at low efficiency. Uninfected D245E6 cells were large polygonal cells with slight overlapping of their edges, which grew in soft agarose but did not release infectious virus. On superinfection with MuLV, D245E6 cells underwent further morphological transformation, and closely resembled MSV infected B/3T3 cells. The superinfected cells released both MSV and MuLV, but their ability to grow in soft agarose decreased. Uninfected KA31 cells were small and highly refractile,

TABLE I. CHARACTERISTICS OF CONTROL AND VIRUS-INFECTED CELLS.

Cell line	Transformed morphology[a]	% Infectious Centers[b] MuLV	MSV	% CFE in soft agarose	Maximum ODC activity (pmoles/10^6 cells)[c]
Balb/3T3	0	<0.1	<0.1	<0.1	49
Balb/3T3 + MuLV	0	78	<0.1	<0.1	63
Balb/3T3 + MSV	+++	100	54	0.8	995
D245E6	+	<0.1	<0.1	16	77
D245E6 + MuLV (transfer 0)	+++	6	9	0.3	509
D245E6 + MuLV (transfer 4)	+++	7	11	5	145
KA31	+++	<0.1	<0.1	15	179
KA31 + MuLV (transfer 0)	+++	94	83	7	356
KA31 + MuLV (transfer 4)	+++	45	38	3	165

[a] Transformed morphology arbitrarily graded as follows: O contact inhibited, nonrefractile cells similar to parent B/3T3; + nonrefractile cells with some cellular overlapping; ++ refractile cells with formation of dense cellular masses; and +++ highly refractile cells with scant cytoplasm and poor anchorage dependency, the cells tended to float into the supernatant fluid prior to reaching confluency.

[b] Percent foci 5 days after plating control or infected cells on A1-2 (MuLV assays) or B/3T3 monolayers (MSV assays).

[c] Maximum ODC activity is the highest measured level of enzyme act.vity, usually occurring four days after infection.

with short spindly processes. They grew in soft agarose before and after superinfection with MuLV, but released MuLV and MSV only after superinfection.

Growth characteristics of the cells employed are shown in Fig. 1. The growth rates of uninfected B/3T3 and D245E6, and MuLV infected B/3T3 were similar, while uninfected and infected KA31 cells grew more rapidly and reached a higher cell density at day 7. As noted previously (5) MSV transformed B/3T3 cells grew slower than uninfected cells, although the differences were not marked in the present experiment, when tissue culture grown virus stocks were substituted for animal tumor harvests. MuLV superinfection of D245E6 cells resulted in a considerable increase in the population doubling time, but had no effect on the growth of KA31 cells. Trypan blue exclusion studies revealed less than 2% nonviable cells in control and virus infected cell lines at all observation points.

ODC levels of cell lines were measured 1, 3, 4, 5 and 7 days after seeding. The highest levels measured (usually occurring 4 days after seeding) are shown in Table I, and the entire curves are presented in Fig. 2. Relatively low ODC levels occurred after seeding and at confluence. Comparable data were obtained when ODC activity was expressed as a function of cell number or of cellular protein. Control B/3T3 and D245E6, and MuLV infected B/3T3 had relatively low 'maximum' levels (ie. the highest levels measured). MSV infection of B/3T3 and MuLV infection of D245E6 cells resulted in 20- and sevenfold increases respectively in maximum ODC activities. Uninfected KA31 cells had a higher baseline ODC activity than the other cell lines, but superinfection resulted in a twofold increase only.

The temporal relationships between elevation of ODC activity, morphological transformation and virus production were also studied. After four passages MSV transformed B/3T3 and MuLV superinfected D245E6 and KA31 cells had not further altered morphologically, or in their ability to release transforming and non transforming viruses, or grow in soft agarose (Table I). However, ODC levels of MSV infected B/3T3 cells fell rapidly after transfer (Fig. 2), while the drop in ODC levels of superinfected D245E6 cells was smaller and took longer. ODC levels of superinfected KA31 cells fell only slightly, but the baseline levels were high and the initial rise on superinfection was modest.

Individual clones of B/3T3 and MSV transformed B/3T3 cells were selected after isolation in liquid or semi-solid media. Characterization of the 13 clones selected and their ODC data are presented in Table II and Fig. 3. Uninfected B/3T3 clones (numbers 1–5) had a flat morphology, did not release viruses, failed to grow in soft agarose, and had low maximum ODC levels. MSV transformed clones 6–12 had higher ODC levels, which appeared related to the degree of morphological transformation. Although isolated from MSV infected cells, clone 13 released only MuLV, had a flat morphology, did not grow in soft agarose, and had low ODC activity. Presumably this clone arose from a cell infected with the nontransforming component of MSV only. With one exception, all clones had maximum ODC activities 4 days after

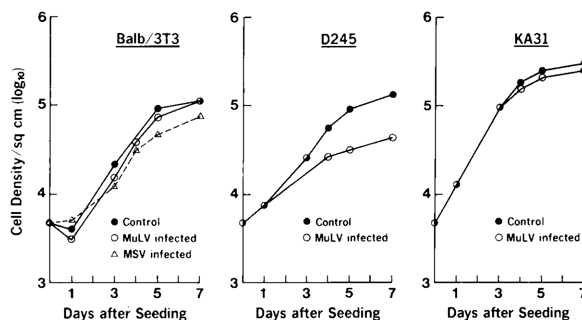

FIG. 1. Growth curves of uninfected and virus-infected cell lines. Cells were infected in suspension with MuLV at a multiplicity (MOI) of 3:1 or MSV (MOI 10:1) at 37° for 1 hr prior to seeding.

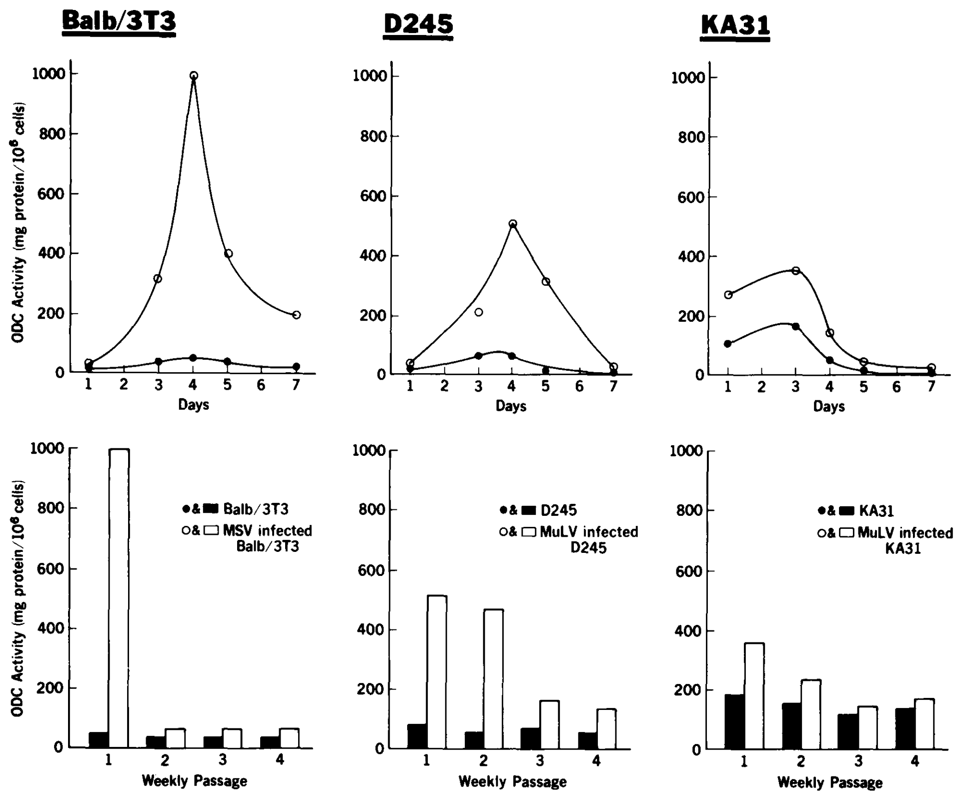

FIG. 2. ODC activity of control and transformed Balb/3T3 cells. In the upper panel, cells were infected immediately prior to seeding and harvested on days 1, 3, 4, 5, and 7. ODC activities in bar graphs (lower panel) represent the maximum levels measured during weekly cell passages.

TABLE II. CHARACTERISTICS OF CONTROL, TRANSFORMED AND VIRUS-INFECTED BALB/3T3 CLONES.[a]

Clone #	Transformed morphology	% Infectious centers		% CFE in Soft agarose	Maximum ODC activity (pmol/10^6 cells)
		MuLV	MSV		
1	0	<0.1	<0.1	<0.1	66
2	0	<0.1	<0.1	<0.1	79
3	0	<0.1	<0.1	<0.1	74
4	0	<0.1	<0.1	<0.1	27
5	0	<0.1	<0.1	<0.1	31
6	+	90	100	0.1	36
7	+	100	100	31	104
8	+++	100	100	0.5	570
9	+++	100	100	0.1	901
10	+++	100	24	69	582
11	+++	100	100	1	522
12	+++	100	76	31	493
13	0	100	<0.1	<0.1	45

[a] Uninfected B/3T3 clones (#1–5) were isolated from liquid medium. MSV transformed clones were obtained from liquid (#7–12) or semi-solid media (#6 and 13). Clones were transferred 18 days after seeding, and analyzed 20 days later. See also legend of Table I.

seeding. The exceptional clone divided slower than the others and was still in exponential growth phase at day 7.

Discussion. While previous studies have indicated that virus induced transformation results in increased intracellular ODC levels, the relationship is complex. Our present experiments further define the association by

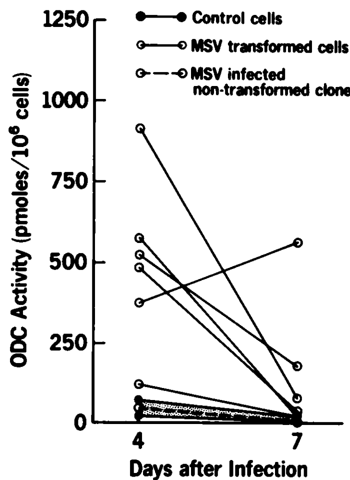

FIG. 3. ODC Activity of control and MSV infected Balb/3T3 Clones. The data of the control clones with the highest and lowest ODC values are displayed. The remaining three control clones had intermediate values lying within the shaded areas. One clone isolated from the MSV infected culture (O- - -O) was nontransformed and only released MuLV. The ODC values of one MSV transformed clone fell within the shaded area and is not represented.

studying several parameters, including morphology, growth rate, virus production and time. ODC levels of transformed and non transformed cells alter with cell growth, the highest levels occurring during logarithmic growth. Elevation of ODC levels (during cell growth) accompanies cellular morphological change to a transformed or more transformed phenotype. Following infection, B/3T3 manifests considerable changes in both morphology and ODC activity, D245E6 more modest alterations, and KA31 essentially none. The elevated ODC levels accompanying transformation cannot be explained by increases in cell growth rates; doubling times (B/3T3 and D245E6) are lengthened or unaltered (KA31) after virus infection. ODC elevation is also not related to release of transforming or non transforming viruses. The cloning experiments indicate that the relatively few transformed clones so obtained have higher ODC levels (during cell growth) than non transformed clones. While transformed clones have a wide renage of ODC activities, acute virus transformation is consistently accompanied by a very high elevation. With cell passage, ODC levels of acutely transformed cells return towards baseline levels, perhaps because most acutely transformed cells fail to divide.

Our findings that elevated ODC levels accompany *acute* virus induced morphological transformation may be explained by the recent report of Isom (16). She found that infection of fibroblasts by potentially oncogenic human cytomegalovirus (CMV) rapidly induced a multiplicity dependent increase in ODC activity. Isom's experiments indicate that CMV infection overrides end product repression of ODC by putrescine. Thus the oncogenic potential of a virus may be related to its ability to interfere with normal regulatory functions of key cellular metabolic enzymes.

Summary. Ornithine decarboxyase (ODC) activity increases when cells are acutely transformed with murine sarcoma virus (MSV). Three contact inhibited or MSV transformed clones of Balb/3T3 were transformed or supertransformed by MSV or its accompanying non-transforming 'helper' virus (MuLV), and the relationships between ODC activity, morphology, virus production and growth rates were examined. Clones isolated from these lines were also studied. All of the virus infected lines released both MSV and MuLV. ODC activities could not be correlated with differences in growth rates. The only consistent relationship was between elevated ODC activity and acute morphological transformation, suggesting that polyamine metabolism plays a crucial role in the transformation process. With time, the elevated ODC activities returned towards baseline levels. Thus ODC activity does not appear to be a useful marker for chronic infection or transformation by type C viruses.

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