Neoplastic development: Paradoxical relation between impaired cell growth at low population density and excessive growth at high density

(spontaneous transformation/carcinogenesis/field effects/epigenetics)

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ABSTRACT   The role of heritable, population-wide cell damage in neoplastic development was studied in the 28 L subline of NIH 3T3 cells. These cells differ from the 17_{3c} subline used previously for such studies in their lower frequency of "spontaneous" transformation at high population density and their greater capacity to produce large, dense transformed foci. Three cultures of the 28 L subline of NIH 3T3 cells were held under the constraint of confluence for 5 wk (5 wk 1° assay) and then assayed twice in succession (2° and 3° assays) for transformed foci and saturation density. After the 2° assay, the cells were also passaged at low density to determine their exponential growth rates and cloned to determine the size and morphological features of the colonies. Concurrent measurements were made in each case with control cells that had been kept only in frequent low-density passages and cells that had been kept at confluence for only 2 wk (2 wk 1°). Two of the three cultures transferred from the 2° assay of the 5 wk 1° cultures produced light transformed foci, and the third produced dense foci. The light focus-forming cultures grew to twice the control saturation density in their 2° assay and 6–8 times the control density in the 3° assay; saturation densities for the dense focus formers were about 10 times the control values in both assays. All three of the cultures transferred from the 2° assay of the 5 wk 1° cultures multiplied at lower rates than controls at low densities, but the dense focus formers multiplied faster than the light focus formers. The reduced rates of multiplication of the light focus formers persisted for >50 generations of exponential multiplication at low densities. Isolated colonies formed from single cells of the light focus formers were of a lower population density than controls; colonies formed by the dense focus formers were slightly denser than the controls but occupied only half the area. A much higher proportion of the colonies from the 5 wk 1° cultures than the controls consisted of giant cells or mixtures of giant and normal-appearing cells. The results reinforce the previous conclusion that the early increases in saturation density and light focus formation are associated with, and perhaps caused by, heritable, population-wide damage to cells that is essentially epigenetic in nature. The more advanced transformation characterized by large increases in saturation density and dense focus formation could have originated from rare genetic changes, such as chromosome rearrangements, known to occur at an elevated frequency in cells destabilized by antecedent cellular damage.

Sublines of NIH 3T3 cells provide models that are useful for studies on neoplastic transformation. In this system prolonged maintenance of cells under the constraint of confluence results in neoplastic transformation as indicated by an increase in saturation density upon passage of the cells and the production

of transformed foci (1, 2). An early accompaniment of the increased saturation density is a decreased rate of proliferation when the cells are passaged repeatedly at low density and frequent production of giant cells in the early postconfluent passages (3). The decreased rate of proliferation becomes more pronounced with repeated rounds of confluence and persists over many generations in low-density passages of the postconfluent cells. Similarly, most cells surviving x-irradiation multiply at a reduced rate for many generations, produce giant cells for a limited time (4–6), and generate some transformed progeny (7, 8), although linkage of impaired proliferation with transformation has not been demonstrated in one and the same experiment. Treatment of cells with carcinogenic polycyclic aromatic hydrocarbons damages cells and gives rise to transformed clones that multiply more slowly at low densities than their untreated nontransformed counterparts but reach higher saturation densities (9). The optimal duration of exposure to the mutagen N-methyl-N'-nitro-N-nitrosoguanidine is similar for cytotoxicity and transformation, but not for mutation, indicating a relation between cell damage and transformation (10). Observations of this type led us to propose that neoplastic development begins with heritable, population-wide damage to cells that decreases their growth rate at low population density and their stability while increasing their saturation density (3). Although the primary effect of carcinogenic treatments in this model is presumed to be nongenetic damage to cells, the accompanying instability increases the probability of genetic changes that can accelerate progression to autonomous growth. We postulated that the correlation between impaired growth at low density and excessive growth at high density reflects a shared, nongenetic cellular lesion; excessive growth at high density could, however, select for cells with genetic lesions that favor and stabilize overgrowth at high density without proportionate impairment of growth at low density. We undertook a study of the relation between impaired growth at low density and transformation with the 28 L subline of NIH 3T3 cells in which transformation is more likely to be expressed in large, dense foci (11) than was the case with the 17_{3c} subline in which the association was first reported (3). The 28 L subline showed the same kinds of evidence of heritable damage and epigenetic regulation found for the 17_{3c} cells. However, what is seen more clearly in the 28 L system is that the more advanced form of transformation is not necessarily associated with a proportionate decrease in growth of cells at low density. This observation lends support to and is consistent with a sequence from epigenetic to genetic change in neoplastic progression.

MATERIALS AND METHODS

The 28 L subline of NIH 3T3 cells was used in this study. It was routinely maintained by weekly standard passages of 400 cells

FIG. 1. Flow chart of successive assays and controls. The three columns on the right represent cultures 1, 2, and 3, which were held for 5 wk in 1° assay in 10% CS and were assayed serially as separate lineages in 2° (A) and 3° (B) assays. Assay A included controls of a 1° assay of the standard passage cells (control A, column 3) and a 2° assay of cells that had been held in a 1° assay in 2% CS for 2 wk (column 4). Assay B had the same two types of control, as indicated in columns 1 and 2.

in 100-mm plastic culture dishes (Falcon) with a growth medium consisting of 90% MCDB 402 (molecular, cellular, and developmental biology 402 medium; ref. 12) and 10% (vol/vol) calf serum (CS). About 200 colonies developed in a week containing an average of $1-2 \times 10^4$ cells per colony. This subline had been through some 110 weekly passages at the beginning of these experiments. Three cultures, designated nos. 1, 2, and 3, were prepared with 10^5 cells in 60-mm culture dishes containing 5 ml of growth medium with 10% CS, which was changed twice a week. They became confluent in 3 days and were incubated for a total of 5 wk, which was designated a 5 wk 1° assay. They were then used for 2° and 3° assays, in each of which 10^5 cells from the preceding assay were seeded on two 60-mm dishes in 2% CS and incubated for two successive periods of 2 wk (Fig. 1). The 2° and 3° assays were accompanied by assays of a control from the standard weekly passage and of cells that had been through a single 1° assay for only 2 wk in 2% CS (2 wk 1° assay). The 2° and 3° assays of cultures from the 5 wk 1° assay included a seeding of only 10^3 cells mixed with 10^5 of the non-focus-forming control cells to obtain a countable number of transformed foci. At the end of each assay, cells of one culture seeded with 10^5 cells were trypsinized and enumerated in a Coulter Counter to give the saturation density. The other culture was fixed with Bouin's reagent, washed, and stained with 4% Giemsa stain.

The growth rates of the cells from the 2° assays of 5 wk 1° cells and from the 2 wk 1° cells were determined by passaging cells at 4×10^4 per 100-mm dish once in growth medium to recover from the direct inhibitory effects of confluence and then transferring 2×10^4 cells per 100-mm dish for a total of

six dishes to initiate exponential growth curves. The standard weekly passages of 28 L cells were passaged in the same way before serving as controls for the growth curves. Two dishes of each kind were counted on each of 3 consecutive days and the increase between 1 and 3 days was used to calculate the rate of exponential growth of the cells. They were also passaged at intervals of 2, 2, and 3 days every week with 4, 4, and 2×10^4 cells, respectively, for a total of 5 wk. The cell yield at each passage was used to calculate the population doublings (PD) per day for that passage (3). Cells from the 2-day recovery period were seeded for colony formation at 10^2 cells per 60-mm dish in growth medium and incubated for 6 days. They were then fixed and stained as above. The area and density of the stained colonies were analyzed by a program developed on a Macintosh II computer using images produced by a Hewlett–Packard Scanjet Plus flatbed optical scanner (13). Density readings on areas of the dish devoid of colonies were subtracted from density readings of the colonies. The average size of the colonies was determined by multiplying their average area by density. Colony parameters (see Table 1) are expressed as the fraction of values for the control cells.

RESULTS

Focus Formation and Saturation Density of Cells Assayed After Prolonged Incubation at Confluence. The appearance of assay cultures from the three categories of cells is shown in Fig. 2. The percentage of cells producing foci and their saturation density are indicated in Table 1. Cells from the standard passage controls produce no foci with the largest number of cells

FIG. 2. Appearance of assays for transformation and colony formation of, left to right: controls, no previous confluence; 2° assays from cells that had been held in 1° assay for 2 wk in 2% CS; 3° assays from cultures 1, 2, and 3 that had been held in 1° assays for 5 wk in 10% CS and 2° assays for 2 wk in 2% CS. The top row was seeded with 10^5 cells, and the second row was seeded with 10^3 cells plus 10^5 control cells. The bottom row was seeded for colony formation with 10^2 cells for 6 days in 10% CS with the same sources of cells used in the assays.

Table 1. Focus formation and growth properties of cells measured after various rounds of confluence

Cell source	% focus formation		Saturation density, cells × 10^{-4} per cm²		PD per day, 1–3 days	Relative colony size (fraction of control± SE)			Colony type (fraction of colonies)		
	Assay A	Assay B	Assay A	Assay B		Area (A)	Density (D)	A × D	Normal	Mixed	Giant
Control	<0.001	<0.001	0.18	0.17	2.16	1.0	1.0	1.0	0.95	0.035	0.015
2 wk 1° 2%	ND	<0.001	ND	0.24	2.08	1.07 ± 0.07	0.95 ± 0.05	1.0 ± 0.12	0.80	0.15	0.05
5 wk 1° 10%	2°	3°	2°	3°	3°*		3°†			3°†	
No. 1	1.9‡	6.2‡	0.40	1.32	1.58	0.71 ± 0.10	0.58 ± 0.04	0.41 ± 0.06	0.39	0.31	0.30
No. 2	1.3‡	5.0‡	0.37	1.00	1.68	0.61 ± 0.09	0.58 ± 0.04	0.35 ± 0.05	0.48	0.21	0.31
No. 3	10.4§	30.0§	2.11	1.84	1.77	0.49 ± 0.03	1.10 ± 0.07	0.54 ± 0.06	0.62	0.19	0.19

Three cultures of 28 L cells were maintained in MCDB 402 with 10% CS for 5 wk with twice-weekly changes of medium. Each of the three (nos. 1, 2, and 3) was then transferred for successive 2° and 3° assays (A and B) of 2 wk each in 2% CS. The 2° and 3° assays were accompanied by 1° assays (2 wk in 2% CS) of control cells from the routine weekly passages of 400 cells in 10% CS and 2° assays of cells harvested at 2 wk from 1° assays of 2 wk in 2% CS, labeled 2 wk 1° 2%. 2° and 3°, secondary and tertiary assays in 2% CS for foci and saturation density originating from the 5 wk 1° assay in 10% CS. ND, not done.
*†Cells from day 14 of 2° assay passaged at low density for 2 days and passaged once again: *, to establish growth curves; or †, to be cloned and scanned 6 days later or examined microscopically for giant cells. Colonies are depicted in Fig. 1 and analyzed in Fig. 3.
‡Light foci. See Fig. 1.
§Dense foci. See. Fig. 1.

(10^5) tested and had a low saturation density, just under 2×10^3 cells per cm² in each of two assays. Cells that had experienced a single 1° assay for 2 wk in 2% CS before reassay also produced no foci, but grew to a slightly higher saturation density than the control cells. Cultures 1 and 2, which had been through a 1° assay for 5 wk in 10% CS, had focus-forming capacities of 1–2% in a 2° assay and 5–6% in a 3° assay. The foci were broad and light as can be seen in Fig. 2 in the seeding of 10^3 cells with 10^5 control cells. Their saturation densities in the 2° assay were about twice that of the control cells and 6–8 times the control value in the 3° assay. The cells of culture 3 had focus-forming capacities of 10% and 30% in the 2° and 3° assays, respectively. The foci were large and dense as can be seen in Fig. 2, and the saturation densities were >10 times that of the control in both assays. The results show discernible transformation in derivatives of all three of the cultures kept for 5 wk in 10% CS, with cells of culture 3 exhibiting the strongest transformation.

Growth Rates of the Cells at Low Population Density. The same cells from the 2° assay of the 5 wk 1° assay that were used for the 3° assay in Table 1 and Fig. 2 were also used to establish growth curves at low density. Cells from cultures 1 and 2 multiplied exponentially at a lower rate than those of culture 3 but all three multiplied more slowly than the cells from the 2 wk 1° assay (Fig. 3; Table 1, "PD per day"). The fastest multipliers were the control cells—i.e., 22–37% faster than the cells from the 5 wk 1° assay.

The persistence of the reduced growth rates of the various postconfluent cultures was evaluated by passaging them serially every 2–3 days at known low density and counting the yield at each passage. The results are presented as an accumulation of PD per day in the graph of Fig. 4. The 2-day points in Fig. 4 of cells taken from the confluent cultures are low because the cells take about 1 day of low-density passage to recover from the constraint of confluence. Cells from cultures 1 and 2 remain at about a 15% lower growth rate than the control cells throughout the 5 wk of frequent passage. Cells from culture 3 grow at about a 10% lower growth rate than controls between days 2 and 7 and at about a 5% lower rate for the rest of the experiment. Thus, the growth rate of the most transformed cells from culture 3 remains higher than that of the less transformed cells of cultures 1 and 2 throughout the experiment.

Growth Characteristics of the Clones. Colonies formed by seeding 10^2 of the same cells used for growth curves are seen in Fig. 2 and the dimensions of the colonies are shown in Table 1. The colonies of cultures 1 and 2, derived from 5 wk 1° cells, are distinctly lighter than colonies produced by the other cultures (Fig. 2), giving a density value just over half that of the others (Table 1). The colonies of culture 3 have a density slightly greater than those of the control and of the cells from the 2 wk 1° assay. The areas of colonies from all three cultures derived from the 5 wk 1° assay are less than the controls but are most conspicuously reduced for culture 3. The overall colony size (area × density) of cells from these three cultures ranges from one-third to one-half that of the control cells and
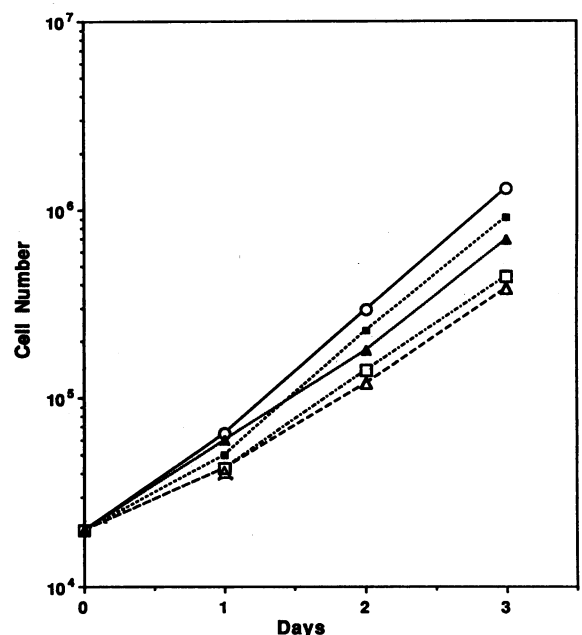

FIG. 3. Growth rates of cells after various pretreatments at confluence. ○, Control cells, no previous confluence; ■, cells that had been held in 1° assay for 2 wk in 2% CS; △, □, and ▲, cultures 1, 2, and 3 that had been held in 1° assays for 5 wk in 10% CS and in 2° assays for 2 wk in 2% CS. All cultures were passaged at low density for 2 days in 10% CS before transfer into 100-mm dishes at 2×10^4 cells in 10% CS to begin the growth curves.
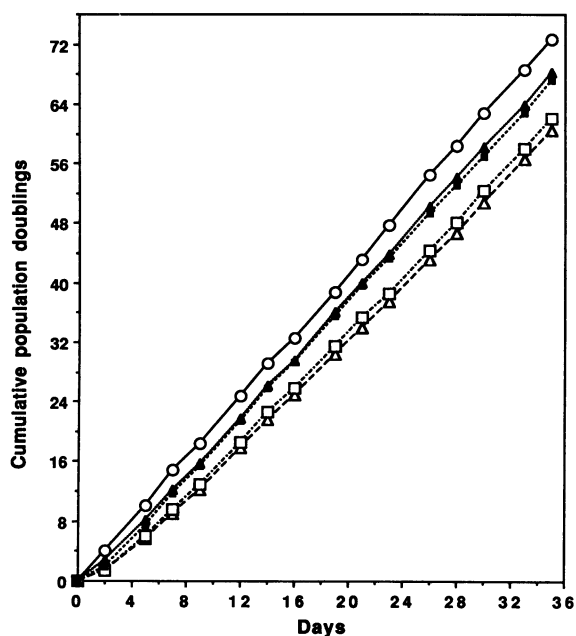
FIG. 4. Cumulative PD of controls and postconfluent cells in repeated low-density passages. Symbols are the same as those in the legend to Fig. 3. The abscissa shows days after beginning low-density passages from confluent cultures.

those from the 2 wk 1° assay. These differences in size are highly significant being >7 times the SEs as shown in Table 1.

Each panel of the scatter diagrams of Fig. 5 compares the distribution of areas and densities of individual colonies from the cultures that had been confluent with those of the controls. In keeping with the calculated results in Table 1, the colonies from the 2 wk 1° cultures display roughly the same distribution of size and density as the controls. The colonies of cultures 1 and 2 show a distinct shift to lower density, with less obvious decrease in area. All the colonies of culture 3 exhibit a decrease in area when compared with control colonies of similar densities.

Production of Giant Cells. Only 5% of the control colonies are mixtures of more-or-less normal-appearing cells with at least three giant cells or consist only of giant cells (Table 1). The number of distinctly abnormal colonies rises to 20% for the 2 wk 1° cells. About 40% of the cells of culture 3 are giant cell producers, and >50% of the cells of cultures 1 and 2 fall in this category. It appears that the highly transformed cells of culture 3 are less damaged by confluence than the less transformed cells of cultures 1 and 2 as judged by growth rates and giant cell production. The fraction of colonies with giant cells decreased to <20% in cells derived from each of the

postconfluent cultures after four low-density passages over a 9-day period of release from confluence (not shown).

DISCUSSION

The 28 L subline of NIH 3T3 cells exhibits evidence of heritable damage associated with transformation similar to that of the 17_{3c} subline (3). There is a persistent slowdown in multiplication of the cells after recovery from the immediate constraint of confluence. Cultures 1 and 2, which had been through 5 wk of confluence in 10% CS and 2 wk in 2% CS, had reduced cell yields for >50 divisions in frequent low-density passages (Fig. 4). Part of the reduced yield may result from increased sensitivity to damage from the trypsinization procedure used to dissociate cells at each passage as demonstrated for cells grown under continuous low-dose radiation from tritiated water (14). The evidence for concurrence in the same cells of impaired growth at low population densities and excessive growth at high densities can be seen in comparing these parameters for cultures 1 and 2 in Table 1. The 2° assay of these cultures gave twice as high a saturation density as the controls, indicating that the bulk of the cells taken from the completed assay had the capacity for overgrowth. This conclusion is borne out by the fact that the cells had six to eight times higher saturation density than the control cells in the 3° assay. The cells from the 2° assay multiplied at only three-fourths the rate of controls at low population densities after a 2-day recovery period. In addition, seeding them at cloning densities produced colonies at 6 days less than half the size of control colonies. Further evidence of damage was the production of a high proportion of giant cells as represented by the incidence of mixed or pure colonies of such cells (Table 1). The results are consistent with Ludford's 1934 finding (15) that malignant cells from transplantable rodent tumors grow more slowly than normal cells, including those derived from the stroma of the same tumors. In the same era, Haddow (16) reported that carcinogenic polycyclic hydrocarbons cause a persistent inhibition of cell growth and proposed this as the primary carcinogenic mechanism of such compounds and of ionizing radiation. He later found that other types of carcinogenic substances produce the same effect (17). Other evidence for a relation between cell damage leading to impaired growth and carcinogenesis has been reported for cells transformed by infection with Rous sarcoma virus (18) and mouse cells transformed by treatment with carcinogenic polycyclic hydrocarbons (9). The results, therefore, support the proposal that a persistent population-wide type of cell damage with associated instability manifest in giant cell production plays a primary role in carcinogenesis.

It should be noted, however, that not all transformed cells multiply more slowly at low densities than their nontransformed progenitors. Smith *et al.* (19) reported that most, but not all, mouse cell clones that had been transformed either
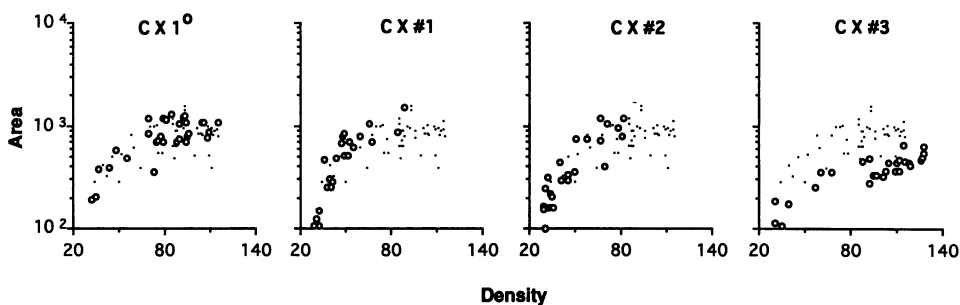

FIG. 5. Area and density of colonies after various pretreatments at confluence. Cells (10^2) were seeded for 6 days in 10% CS. The same set of control colonies appears as dots in each panel. The open circles represent cells as follows: 1°, a primary assay for 2 wk in 2% CS; #1, #2, and #3, three cultures that had been held for 5 wk in 10% CS and 2 wk in 2% CS. The colonies analyzed are those photographed in the bottom row of Fig. 1.

"spontaneously" or by chemical treatment are slow growers. In the present case we find among the three cultures derived from the 5 wk 1° assay that the cells of culture 3 multiplied substantially faster than those of cultures 1 and 2, although the former produced much denser foci than the latter in assays. Culture 3 also recovered from confluence more quickly in low-density passages than did cultures 1 and 2 (Fig. 4) and produced fewer giant cells (Table 1). Isolated colonies of culture 3 were at least as dense as those of the control and much denser than those of cultures 1 and 2. Though we have proposed that impaired growth at low density and excessive growth at high density result from a common lesion in the cells, this is unlikely to be the case for advanced transformation like that of culture 3. While the early increases in saturation density appear to occur in a large fraction of treated cells, as does the impaired growth, the advanced transformation occurs only in a very small fraction of cells (2, 8, 20) that is rapidly expanded by selective growth at confluence. Although the early stages appear to be epigenetic, the later ones could very well be genetic in origin. Since the number of advanced transformants produced by x-irradiation is not increased by treatment with mutagens, it is unlikely that they arise from simple point mutations (8). Genomic rearrangements, however, are commonly detected in cells transformed by x-rays, ultraviolet light, and 3-methylcholanthrene (21). They are also a common occurrence in mouse and hamster cells after explantation to culture (22–24). Prolonged incubation of primary human cells at confluence results in a large variety of chromosomal aberrations associated with escape from contact inhibition and a prolonged life-span (T. Ignatova, personal communication). Similar treatment of near-diploid Chinese hamster ovary cells produces abnormal nuclear morphology with multiple nuclei and micronuclei (C. Vidair, personal communication). Chromosomal instability is prominently associated with progression in human melanoma (25) and cancer of the esophagus (26) and is thought to play a major role, generally, in human cancer (27, 28). Chromosomal changes, therefore, have to be given serious consideration in the progression of cells to advanced transformation.

It should be emphasized, however, that the early changes observed in our cells have all the earmarks of epigenetic change. They are associated with evidence of damage in a large fraction of the population (3). The early increases in saturation density of cells transferred after prolonged confluence also occur in a large fraction of the population in a uniform, graded manner before dense foci appear (2, 20). Evidence of population-wide damage to cells is also seen after x-irradiation (6) and is followed by elevated frequencies of certain complex types of mutation associated with the hemizygous *HPRT* locus (29) but no increase in specific point mutations (30). The evidence for epigenetic change is not restricted to cells adapted for culture. Treatment with x-rays or chemical carcinogens of mammary epithelium or thyroid cells immediately after their removal from mice followed by reinoculation into mice results in 1 cancer per 10–300 clonogens (31, 32). Such frequencies of cancer development are orders of magnitude higher than expected from local mutations, and they are likely to be underestimates of the proportion of cells that undergo early, incipient stages of neoplastic development (2, 20). The theme of heritable damage, possibly induced by free radical damage to lysosomal membranes (3), provides a plausible basis for the population-wide early changes. It would explain the evidence for abnormalities found in normal-appearing tissue at a distance from human cancers of the bladder and colon (33, 34).

All the evidence points to the origin of cancer from a field of altered, unstable but normal-appearing cells (35) rather than from isolated mutations among otherwise unaltered cells.

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