Growth Rate and Chromosome Number of Tumor Cell Lines With Different Metastatic Potential

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We investigated whether the metastatic potential of various tumor cell lines
was related to chromosome counts or to rate of growth in vitro or in vivo.
Clones of known metastatic potential derived from a C3H⁻ fibrosarcoma induced
by UV radiation (UV-2237) and from C57BL/6 B16 melanoma were tested
for these characteristics. No correlation was found between the growth rate
of these clones in monolayer culture or at a subcutaneous site and their
ability to produce metastases. The cells from clones of UV-2237 were mainly
in the diploid range with only one exception, and the B16 clones were all
hyperploid. Thus, there was also no correlation between malignant behavior
of the clones and gross changes in chromosome number.

Key words: metastatic potential, growth rates, chromosome number and range

Clinical and morphologic observations of human neoplasia have suggested that
tumors may progress from benign to malignant behavior over a period of time [1—4].
The progression of evolution of a tumor has been attributed to the emergence of new
variant cells that have a selective advantage for growth in vivo. It has been suggested that
such variants have an increased growth rate and that they also have an abnormal number
of chromosomes [5—8]. In general, benign (noninvasive, nonmetastasizing) tumors are
thought to be well-differentiated and to grow slowly; mitotic figures are infrequent, and
those present are usually normal. In contrast, malignant (metastasizing) tumors are
usually undifferentiated and consist of a large number of dividing cells. These dividing
cells may have many abnormal chromosomes and higher chromosome numbers, and they
may exhibit varying degrees of anaplasia [4].

Despite numerous observations, it is still unclear whether an increased growth rate
or change in chromosome number are requisite for the progression of tumors from a
benign to a malignant state. This issue cannot be resolved by morphologic examination of
clinical specimens, nor can studies be performed on a variety of tumors of different
histologic types and of possible different etiologies obtained from different donors. In-
stead, studies must be performed on neoplastic cell lines isolated from a single neoplasm

that have defined biologic behavior in vivo. Recent cloning studies demonstrated that two murine neoplasms, the C57BL/6 B16 melanoma [9] and a C3H⁻ fibrosarcoma induced by ultraviolet (UV) radiation [10] are heterogeneous and contain subpopulations of cells with differring capacities for metastasis. These clones breed true upon recloning. The availability of these clones, which vary in their metastatic behavior in vivo, affords us an opportunity to investigate which properties of cloned tumor cell populations are associated with metastasis. In these experiments we wished to determine whether the metastatic potential of a variety of tumor cell clones was associated with an abnormal chromosome number and/or with a rapid growth rate, as measured in animals or in cell cultures.

MATERIALS AND METHODS

Animals

Specific-pathogen-free C57BL/6 and C3H/HeN (MTV⁻) (C3H⁻) mice were obtained from the Animal Production Area of the Frederick Cancer Research Center. Within a single experiment all mice were age- and sex-matched.

Cell Cultures

The B16 melanoma, which arose in a C57BL/6 mouse, was established in culture as described previously [11, 12]. In the present studies we used in vitro cloned lines derived from our B16 parent culture [9]. The UV-2237 is a fibrosarcoma that was induced by chronic UV irradiation of a female C3H— mouse. The tumor was established in culture from the first in vivo passage in immunosuppressed syngeneic mice, and cells of the sixth in vitro passage were cloned as previously reported. All cell lines were grown as monolayers on plastic flasks in MEM Autopow medium (Flow Laboratories, Rockville, Maryland) supplemented with 10% fetal calf serum (FCS), glutamine, nonessential amino acids and vitamins designated as complete minimum essential medium (CMEM) (Grand Island Biological Co., Grand Island, New York). The cultures were maintained at 37°C in a humidified incubator in an atmosphere containing 5% CO_2. All cell lines were examined and found free of Mycoplasma [13] and the following murine viruses: reovirus type 3, pneumonia virus of mice, K virus, Theiler's virus, Sendai virus, minute virus of mice, mouse adenovirus, mouse hepatitis virus, lymphocytic choriomeningitis virus, ectromelia virus, and lactate dehydrogenase virus (Microbiological Associates, Walkersville, Maryland). In order to assure reproducibility between in vivo and in vitro assays the cultures were tested within 2 weeks after recovery from frozen stocks.

The tumor cells were harvested from subconfluent cultures (50–70% confluent) by rinsing the monolayers with 0.25% trypsin–0.02% versene solution. After 1 min, the flasks were tapped sharply to dislodge the monolayers, and the cells were washed in CMEM with 10% FCS. The cells were resuspended in Hanks's balanced salt solution (HBSS) for counting and injection. Only suspensions containing single cells of > 90% viability were used for injection.

Experimental Pulmonary Metastasis

Unanesthetized mice were inoculated IV via the tail vein with 0.2 ml of 5×10^4 (B16) or 1×10^5 (UV-2237) viable single tumor cells suspended in HBSS. All mice were killed 18 days after tumor cell injection, and their lungs were removed, rinsed in water, and fixed in formalin (B16) or Bouin's fixative (UV-2237). The number of lung tumor colonies was determined by counting surface metastases under a dissecting microscope,

since most experimental metastases in mice are located at the surface of the lung [14].
Metastases were counted in a blind fashion by two observers.

In Vitro Growth Rate Determinations

Cell lines were plated at a density of 10^4 cells per 60-mm plastic petri dish (Falcon
Plastics, Oxnard, California). Duplicate cultures were trypsinized, and the number of cells
per dish was determined every 24 h for 5 days with a Coulter Counter (Coulter Electronics,
Inc., Hialeah, Florida).

Tumor Growth in Vivo

C3H$^-$ mice (8 weeks old) were injected subcutaneously with 10^6 cells of the UV-
2237 fibrosarcoma clones (5 mice per group). The tumors were measured with a caliper once
a week in three diameters, and the average tumor diameter was determined for each animal.

Chromosome Analysis

Tumor cells were plated at 10^6 per 100-mm plastic petri dish, and 24–36 h later
the cultures were incubated with 1 μg/ml Colcemid (Calbiochem, La Jolla, California).
At the end of 2 h at 37°C, the cells were trypsinized (0.25% trypsin–0.02% EDTA),
centrifuged, and resuspended in 0.075 M KCl for 45 min. Samples were fixed three times
in methanol-acetic acid (3:1) and then resuspended in this fixative. The fixed cells were
dropped onto slides. Chromosome counts from at least 100 cells were made on each cell
line. The differences in chromosome numbers were analyzed by Student's t test, the
Mann-Whitney U test, and the Median test.

RESULTS

Tumor Growth Rate vs Metastatic Potential

Clones of the B16 melanoma were classified as having low, intermediate, or high
metastatic potential. These categories were based on the number of macroscopic tumor
colonies present in the lungs of syngeneic mice injected IV 18 days earlier with 5×10^4
tumor cells. The clones of low metastatic potential (clone numbers 16, 15, and 12) pro-
duced less than a median of seven lung colonies in groups of ten recipients, the inter-
mediate parent B16 line gave 40 lung colonies, and the highly metastatic clone (number 9)
produced more than 500 tumor colonies per recipient (Table I).

Clones of the UV-2237 fibrosarcoma were also classified as having low, intermediate,
or high metastatic potential based on the number of tumor colonies present in the lungs
of mice injected IV 18 days earlier with 1×10^5 tumor cells. In this tumor system the
formation of lung metastases correlates with the formation of extrapulmonary metastases
and with the formation of spontaneous metastases from subcutaneously growing tumors
[10]. The clones of low metastatic potential (numbers 15 and 38) produced less than a median
of two lung colonies in groups of ten recipients. The highly metastatic clones (numbers
39 and 25) produced more than 135 tumor colonies per recipient. Intermediate clones
form a continuous series between clones which are obviously "low" and those which are
obviously "high."

In spite of the wide variation of metastatic behavior of the B16 or UV-2237 clones,
the doubling times of these cell lines in monolayer culture did not differ significantly
from each other (Tables II, III). For UV-2237 clones, the doubling times ranged from 17
to 24 h, and there was no positive correlation between short doubling time and high
metastatic potential (Table III).

TABLE I. Quantitative Lung Colony Assay With B16 Melanoma or UV-2237 Fibrosarcoma Clones

Syngeneic[a] recipients	Tumor source	Median number (range) of pulmonary metastases
C57BL/6	B16 parent[b]	40.5 (8–131)
	Clone 15	5 (2– 20)
	Clone 16	3.5 (0– 15)
	Clone 12	6 (0– 34)
	Clone 9	>500
C3H–	UV-2237 parent[c]	160.5 (17–300)
	Clone 38	2 (0– 8)
	Clone 15	1 (0– 9)
	Clone 42	5 (0– 39)
	Clone 43	9.5 (0– 78)
	Clone 26	25.5 (4–212)
	Clone 39	135 (85–248)
	Clone 25	140.5 (79–300)

[a]10 mice/group. Mice were injected IV and killed 18 days later. The number of lung tumor colonies was determined with a dissecting microscope.
[b]5×10^4 viable cells injected IV (see Fidler and Kripke [9]).
[c]1×10^5 viable cells injected IV (see Kripke et al [10]).

TABLE II. In Vitro Growth Rate of B16 Melanoma Parent and Cloned Lines of Varying Metastatic Potential

Tumor lines	Metastatic potential[a]	Doubling time[b] (hours)
B16 Clone 16	Low	12.4
B16 Clone 15	Low	13.5
B16 Clone 12	Low	12.2
B16 Parent	Intermediate	12.4
B16 Clone 9	High	12.4

[a]Based on the number of pulmonary tumor colonies present at 21 days in mice injected IV with 5×10^4 cells [9].
[b]Doubling times calculated form the slope of a 96-h growth curve of cells in logarithmic growth. The differences in doubling time were not significant within a confidence limit of 95%.

Since the growth rate of tumor cells in culture need not reflect the rate of growth of the cells in an animal host, we also measured the growth rate of the tumors at a subcutaneous site in syngeneic mice. Again there was no direct relationship between the average tumor size at various weeks and the metastatic behavior of the tumors (Table III). Clones of low metastatic potential also exhibited a slow growth rate subcutaneously; however, the rapid growth of tumors subcutaneously was not necessarily associated with high metastatic potential. For example, clone 25, which is highly metastatic, grew more slowly than the less metastatic clone 43.

TABLE III. In Vitro and In Vivo Growth Rates of UV-2237 Fibrosarcoma Clones of Different Metastatic Potential

Clone No.	Metastatic potential[a]	Doubling time[b]	Tumor incidence at following weeks after subcutaneous injection[c]		
			1	2	3
38	Low	17.4	0/5 (0)	0/5 (0)	1/5 (1 ± 1)
15	Low	20.3	0/5 (0)	1/5 (2.2 ± 0.9)	3/5 (3.1 ± 1.3)
43	Intermediate	18.7	4/5 (4.5 ± 1.1)	5/5 (8.0 ± 0.6)	5/5 (11.1 ± 0.6)
39	High	21.7	0/5 (0)	5/5 (5.4 ± 0.6)	5/5 (9.4 ± 2.4)
25	High	24.4	1/5 (0.8 ± 0.8)	3/5 (2.8 ± 1.1)	5/5 (4.8 ± 1.1)

[a]See text for details.
[b]Doubling times in hours calculated from the slope of a 120-h growth curve (cells in logarithmic growth). There were no statistically significant differences among the clones within a confidence limit of 95%.
[c]Average tumor diameter in mm ± SE measured at weekly intervals after the subcutaneous injection of 10^6 cells into syngeneic mice (five mice per group).

Chromosome Patterns vs Metastatic Potential

To determine whether clones of different metastatic potential also differed in chromosome number, we processed clones of UV-2237 and B16 for chromosome counts. Typical chromosome spreads are shown in Figure 1. Clone 43 of UV-2237 is shown at the top of Figure 1 and clone 15 of B16 is at the bottom. None of the UV-2237 clones show gross chromosomal abnormalities. All of the B16 clones examined have four abnormal chromosomes which are metacentric. The chromosome number of several B16 melanoma clones and parental tumor was examined. The mode and range of chromosome number was very similar. For example, for the parental tumor the mode was 79 with a range of 65–88. For clone 15 (low metastatic) the mode was 74 and chromosome range was 68–80. For the highly metastatic clone 9, chromosome mode was 75 and the range was 67–80.

Since the UV-2237 clones are very stable (at least up to 2 months of continuous culture) with regard to their metastatic behavior in vivo (Cifone, unpublished data), extensive chromosome studies were carried out with these clones. One hundred or more spreads were counted per clone. All spreads of a given clone were prepared at the same time from cultures grown for no longer than 7 days after recovery from frozen stocks. The mode and range of the chromosome numbers of the lines are shown in Table IV. With one exception (clone 38), neither the modes of the different clones (which were very similar) nor the differences in ranges (as seen in column 4) correlate with metastasis. Clone 38 exhibits a very different chromosome pattern from the others, ie, hyperploidy, with a wide range in chromosome number (Fig. 2c). Nonetheless, clone 38 is virtually nonmetastasizing. The ranges in chromosome number from at least 100 cells of clones 25 and 15 (highest and lowest metastatic potential, respectively) are indistinguishable, as shown in Figure 2. The data for the UV-2237 clones were analyzed statistically, and no individual clone differed significantly from the general population of all clones (except clone 38) by either the Student t test, the Mann-Whitney test, or the Median test.
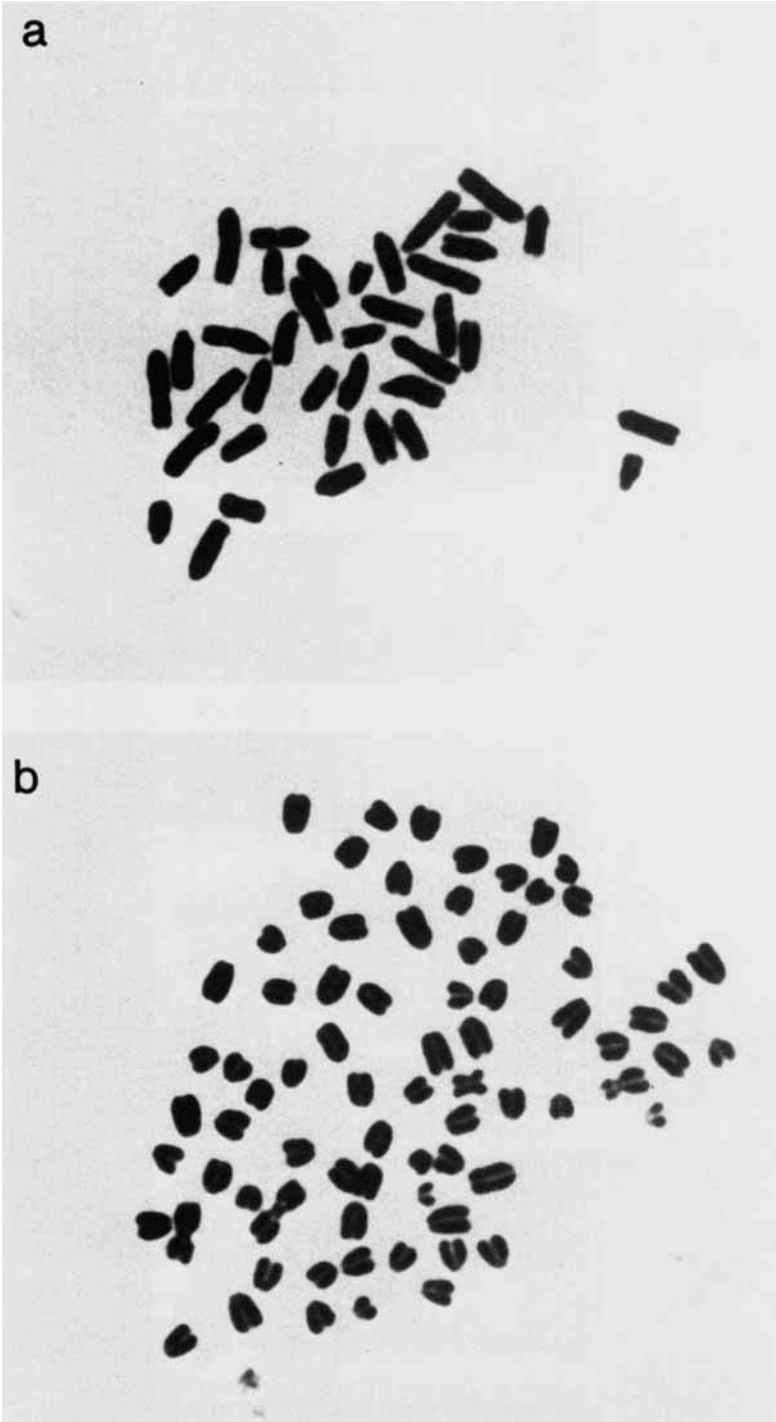
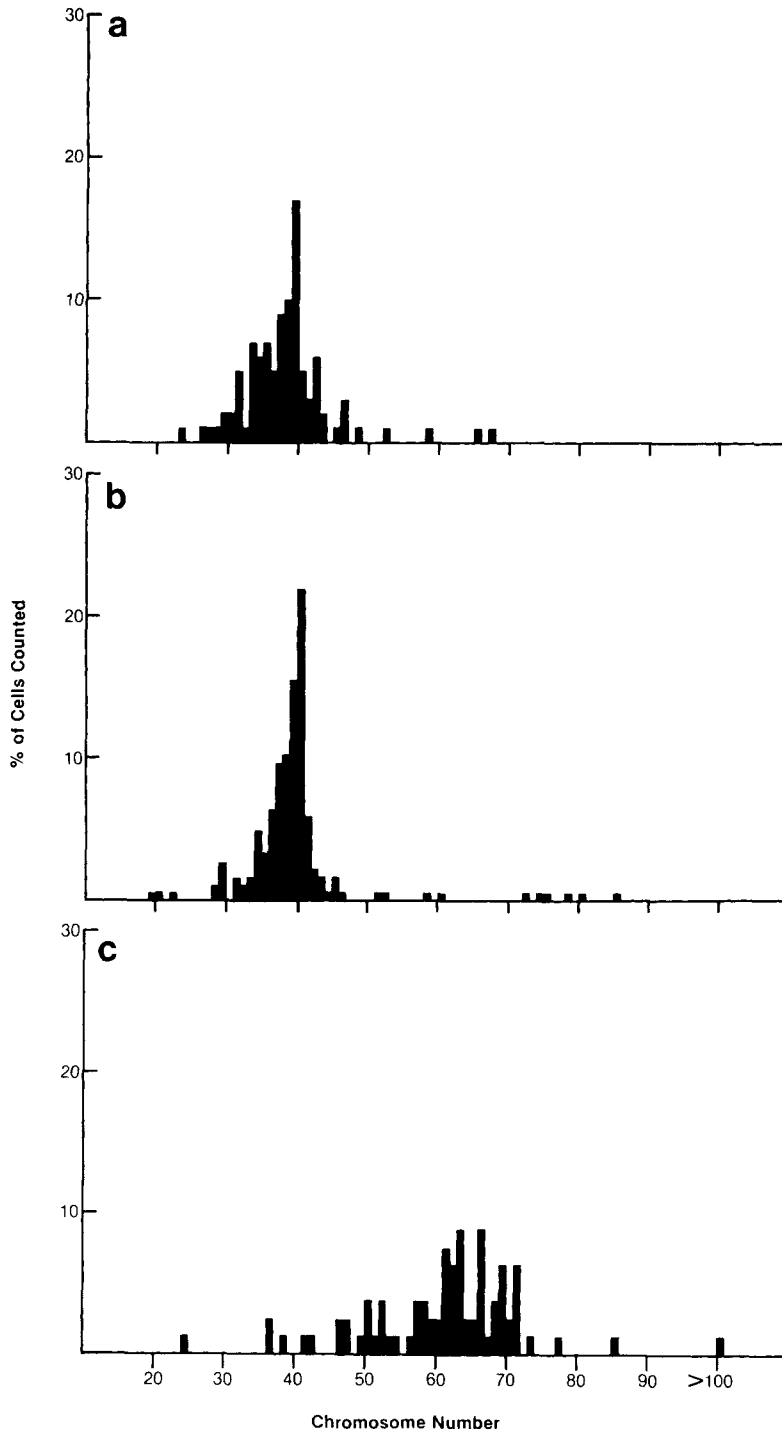Fig. 1. Metaphase of UV-2237 and B16 lines. a: UV-2237 clone 43 (× 1,000); b: B16 clone 15 (× 1,000).

Fig. 2. Distribution of chromosome numbers of three UV-2237 clones. a: UV-2237 clone 15; b: UV-2237 clone 25; c: UV-2237 clone 38.

TABLE IV. Chromosome Mode and Range of UV-2237 Fibrosarcoma
Clones With Different Metastatic Potential

| Clone No. | Metastatic potential | Percentage of cells at the mode | |
		Chromosome mode[a]	Chromosome range[a]
15	Low	39	23−68
38	Low	61−66	24−> 100
42	Low	40	21−71
31	Intermediate	39	27−72
26	Intermediate	41	19−63
25	High	40	19−85

[a]Average of 100 or more chromosome spreads per clone. Where no
definite mode is apparent, a range where the highest percentage of
chromosomes occurs is given.

DISCUSSION

These studies were designed to evaluate whether the metastatic potential of cloned
murine tumor cell lines correlated with a deviation in chromosome number and/or an
accelerated growth rate in vivo or in vitro. Many investigators have attempted to deter-
mine whether a change in chromosome number from diploidy is associated with the
neoplastic malignant transformation of normal cells. Although the DNA content and
chromosome number of neoplastic cells often differ from that of normal cells, such
changes are by no means characteristic of all tumors. In fact, in some tumor cells no such
abnormalities can be demonstrated [5−8]. In some systems chromosome alterations also
occur in the later stages of tumor progression [6, 7, 15, 16]. Even with tumors that were
found to be aneuploid initially, additional chromosome changes were observed that were
associated with an increased growth rate [6, 7, 17]. Thus, a change in chromsome num-
ber from diploidy to aneuploidy has been implicated in the progression of tumors to a
malignant stage. The progression to malignancy, however, is not always associated with an
increase in mean chromosome number. The chromosome number of cells obtained from
different patients with either dysplasia, carcinoma in situ, or invasive carcinoma of the
cervix was determined, and a decrease in the mean chromosome number was associated
with the progression of tumors from carcinoma in situ to invasive carcinoma [16].

Our analysis of the chromosome number of cloned populations of cells with
differing metastatic potential reveals no correlation between metastasis and chromosome
number and range. The one case of hyperploidy, observed in cells of UV-2237 clone 38,
was not associated with a high metastatic potential, since clone 38 does not metastasize.
Further, hyperploidy was not required for nonmetastatic behavior, because UV-2237
clones 15 and 42, which are also nonmetastasizing, are near diploid. In the case of the
B16 melanoma, cells with either low or high metastatic potential were hyperploid. Thus,
systematic alterations in chromosome number were not associated with metastatic
behavior in the two tumor systems that we studied. It is important to note that the
analysis used here (chromosome counts) is indeed gross. Systematic changes in chromo-
somes of metastatic cells could perhaps be demonstrated by more sophisticated techniques
such as chromosome banding. In some tumor systems, such as murine lymphoma induced
by radiation leukemia virus [18] and a murine T-cell leukemia induced by 7, 12-

dimethylbenz-(a)-anthracene [19], constant chromosome changes, ie, trisomies No. 15, No. 17, and trisomy No. 15, respectively, were associated with neoplastic cells. In contrast, consistent chromosomal changes were not reported in earlier studies of nine primary and 14 transplanted rat leukemias and lymphomas [20]. Therefore, if changes more subtle than chromosome number and range are indeed associated with increased malignancy, they were not detected here.

An increased growth rate could be responsible for the eventual dominance of tumor variants with an increasingly malignant character. We tested this possibility by measuring the growth rates in vitro of a variety of cell lines from two different tumor systems and comparing them with the metastatic behavior of these tumor lines. No correlation was detected between these parameters in either tumor system. However, measuring the doubling time of cells in vitro does not take into account the possible involvement of host factors that could alter the rate of tumor growth. For this reason, we also measured the rate of growth of several UV-2237 clones in syngeneic mice. As expected, there was no direct correlation between growth rate of a cell line in vivo and its doubling time in vitro. Furthermore, there was no simple relationship between metastatic behavior of the cell lines and their growth rate in animals. Although the nonmetastatic clones grew slowly in animals after subcutaneous injection, the highly metastatic clones did not necessarily grow more rapdily in vivo.

In summary, we did not find a correlation between increasing malignancy and gross changes in chromosome number or tumor growth rate. Although these parameters may be associated with malignant progression in some instances, it does not appear that such changes are requisite for the progression of murine fibrosarcoma or melanoma tumors from a benign to a malignant phenotype.

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