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Increased Tumorigenicity of Polyploid Ehrlich Variants During Growth *in vivo* is Associated with Karyotypic Changes

YATAKA MATSUYA and ISAO YAMANE

Department of Cell Biology, Research Institute for Tuberculosis and Cancer, Tohoku University, 4-1, Seiryo-machi, Sendai 980, Japan

Abstract—*In previous studies, treatment of Ehrlich ascites tumors (EAT) with either Sendai virus, Herpes virus or neuraminidase produced variants that differed significantly from EAT in both tumorigenicity and karyotype.*

In this study, less tumorigenic variants of EAT were isolated by culturing in media containing low concentrations of serum or without serum. In contrast to the relative stability of tumorigenicity in such variants cultured in vivo, *the tumorigenicity of these variant clones passaged* in vivo *was variable, depending on the variant clone. During growth* in vivo, 3 *variant clones independently isolated from serum-free medium, low serum-containing medium and after neuraminidase treatment produced markedly heterogeneous tumors, and after* 4 *passages the tumors were about* 1000 *times more tumorigenic than the initial variants. In generating new tumors, the tumors always segregated chromosomes. The banded chromosome analysis of a variant clone isolated from serum-free medium and its malignant tumor showed that most of the chromosomes segregated from the clone were normal types. On the other hand, the tumorigenicity of Herpes virus-induced variants was unchanged during growth* in vivo.

INTRODUCTION

THE pioneering studies of Fidler, Poste and their colleagues [1–4] have shown that many experimental animal tumors contain subpopulations of cells that differ widely in both tumorigenicity and metastatic ability. This cellular heterogeneity is believed to result from the emergence of variant tumor cells with altered metastatic properties during progressive tumor growth [5, 6]. The composition of the original uncloned tumor population remains constant during passage *in vivo*, in contrast to the instability of cloned populations [2, 7]. The mechanisms underlying the formation of variant subpopulations of tumor cells are poorly understood.

In previous studies, we found that after treatment of Ehrlich ascites tumor cells (EAT) with either Sendai virus [8], Herpes virus or neuraminidase (manuscript in preparation), variant cells appeared in the culture and that all of these variants were less tumorigenic and had more chromosomes than the original EAT. In addition, we isolated less tumorigenic variants, without pretreatment of EAT, by culturing EAT in media containing low serum or no serum.

This paper describes the stability of tumorigenicity in each of various variants maintained *in vivo* and *in vitro*. In many of the variant types, serial passage *in vivo* results in a rapid increase of tumorigenicity that is accompanied by a loss of chromosomes from each variant.

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MATERIALS AND METHODS

Cell

Ehrlich ascites tumors (EAT) were maintained by weekly intraperitoneal (i.p.) transplantation into ddi strain of mice. Variants with reduced tumorigenicity were isolated after treatment of EAT with Sendai virus [8], Herpes virus or neuraminidase (manuscript in preparation). These variants as well as the control were cultured in F12 medium with 2 times the concentration of amino acids and vitamins of Eagle's MEM (enriched F12 medium) and 10% calf serum (CS). To ensure the reproducibility of the *in vivo* assays, these variant clones were used within a month of culture after recovery from frozen stocks.

Isolation of variants capable of proliferating in serum-free medium or medium containing low concentrations of serum

EAT were withdrawn from the peritoneal cavity of mice who had been injected 8–10 days earlier. The ascites tumors were suspended in Hanks' balanced salt solution (HBSS) and centrifuged. The cell suspension was washed twice with HBSS and resuspended in HBSS at a concentration of 2×10^7 cells/ml. Each of 10^7 cells suspended in HBSS was plated in a 60 mm dia. tissue culture dish (Falcon) containing 5 ml of enriched F12 medium supplemented with a reduced amount of sodium bicarbonate (0.05%) and either 0.05% bovine serum albumin (Cohn's fraction V) or 1.2% CS. Cultures were maintained at 37°C in a humidified 5% CO_2 incubator and the medium was renewed twice a week. The frequency of variant formation was < 1 in 10^7 EAT.

Cloning procedure

Attached cells were gently but rapidly trypsinized. Cells were suspended at a concentration of 5 cells/ml in enriched F12 medium containing 10% CS and 0.2 ml was plated into each well of a Microtest II plate (Falcon). The problem of cell clumping in the cells to be cloned was minimized by frequent stirring of cell suspension and by careful microscopic examination of the suspension to be certain that it was free from aggregated cells. The plate was incubated at 37°C in a 5% CO_2 incubator for 9 days. The plate was inspected visually with an inverted microscope. Inspection was performed after seeding 24 hr later and wells containing 1 cell were marked. Cloning was repeated to ensure the homogeneity. The plating efficiency was > 80%.

Transplantability test

The transplantability of these variants was tested by i.p. injection into 3–4-week-old ddi strain mice. Animals were observed for 4 weeks, and the take incidence was assessed from the number of mice bearing ascites tumors.

The dose that produced tumors in 50% of the mice (TFD_{50}) was calculated by the method of Kärber [9].

Chromosome preparation

Chromosome preparation was made by the air-drying method after colchicine and KCl treatment. The slides were stained with Giemsa for routine karyotyping. For the banding pattern analysis, Giemsa-staining with trypsin [10] was used.

RESULTS

Stability of tumorigenicity in the control and a variant cultured for a long period

Both the control and a Sendai virus-induced variant (HVJ-X) were cultured serially after cloning (Table 1). The tumorigenicity and the number of chromosomes of the control were stable over the 15-month time period *in vitro*, whereas the HVJ-X clone showed gradual decrease in both tumorigenicity and the number of chromosomes with time *in vitro*.

Table 1. Change in tumorigenicity and chromosome number in control and Sendai virus-induced variants during serial culture in vitro

Clone	Time in culture (months)	Tumorigenicity (log TFD_{50})	Total No. of chromosomes* (mean ± S.D.)
Control	—	1.5	68 ± 3
	5	1.5	66 ± 3
	9	1.6	67 ± 3
	15	1.6	66 ± 4
HVJ-X	—	5.0	88 ± 3
	5	5.6	85 ± 4
	9	5.9	80 ± 3
	15	6.2	77 ± 4

*At least 50 metaphases were counted.

Instability of tumorigenicity in various variant clones serially passaged in vivo

Variants with lower tumorigenicity have been isolated after treatment of EAT with or without agents capable of inducing variants ([8], manuscript in preparation). These variants were cloned and some clones were immediately frozen. Among these variants, clones of Sendai virus-induced (HVJ-X or-2), Herpes virus-induced (HS-5-1), neuraminidase-induced (NA-1), serum-free medium grown (SF-74A) and 1.2% CS-supplemented medium grown (1.2S-B), as well as the control EAT were used for the experiments of serial passage *in vivo*. Cells from these clones were injected i.p. into mice. The ascites tumors obtained from tumor-bearing mice were again injected into mice, without any culture *in vitro*. This procedure was repeated several times. The results are summarized in Fig. 1A. Among these variants, the tumorigenicities of the control and HS-5-1 were observed to be stable even after almost 4 months *in vivo*.

In contrast to these 2 lines, the other variants became rapidly more malignant during growth *in vivo*, suggesting that there was selection *in vivo*. The TFD_{50} values of clones, NA-1, SF-74A and 1.2S-B, decreased rapidly for 2.5 months, at which time they were almost as tumorigenic as the control. The change in tumorigenicity of HVJ-2 was different from that of these 3 variants; the TFD_{50} values of HVJ-2 decreased rapidly for 1.5 months at approximately the same rate as these other variants but thereafter the TFD_{50} stabilized. The biphasic pattern of change in tumorigenicity was not unique

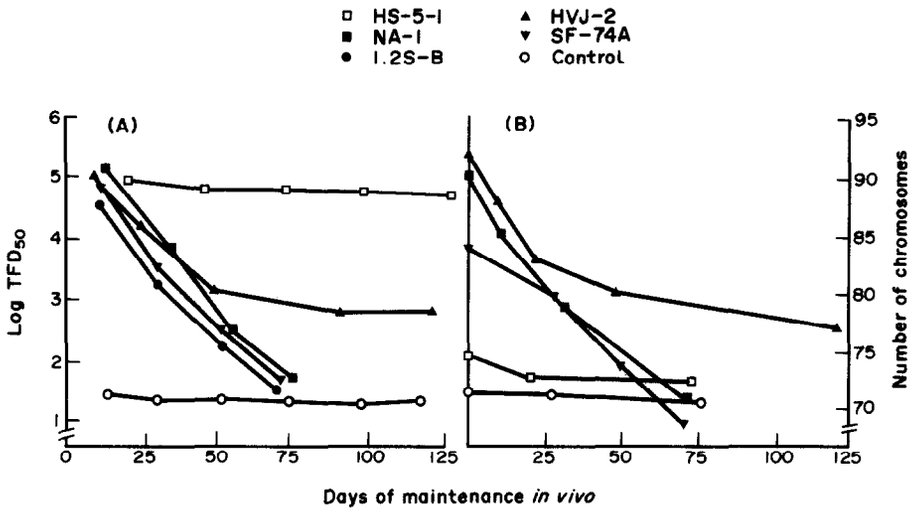

Fig. 1. Changes in TFD_{50} and chromosome numbers in control and various variant clones during growth in vivo. *A, correlation beween TFD_{50} and days of maintenance* in vivo; *B, correlation between chromosome numbers and days of maintenance* in vivo.

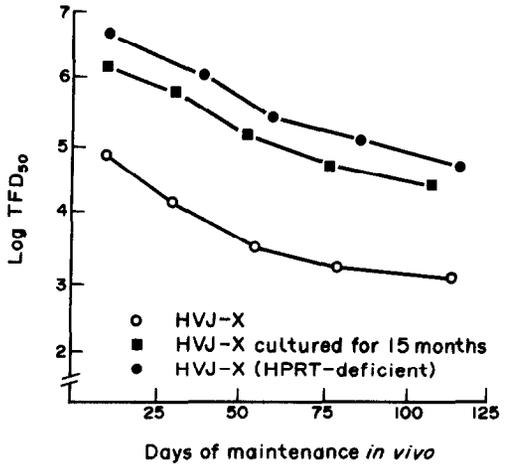

Fig. 2. Changes in TFD_{50} of Sendai virus-induced variant clone and its derivatives during growth in vivo.

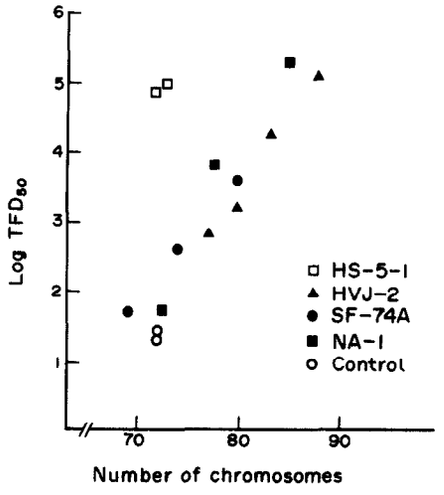

Fig. 3. Correlation between TFD_{50} and chromosome numbers of control and various variant clones during growth in vivo.

for HVJ-2. Similar qualitative results were observed for HVJ-X cells, before or after culture *in vitro* for 15 months, and with its hypoxathine phosphoribosyltransferase (HPRT)-deficient mutant (Fig. 2). This mutant was isolated in a single-step selection by exposing 10^7 HVJ-X cells to 3 μg of 6-thioguanine/ml. The deficiency in HPRT was confirmed either by low [^{3}H]-hypoxanthine incorporation or by the absence of growth in HAT medium [11]. The rate of decrease in the TFD_{50} of these HVJ-X lines was similar to each other, even though each had different initial TFD_{50} values (Fig. 2) and was lower than that of HVJ-2 (Fig. 1A). These results suggest that the patterns of change in tumorigenicity of Sendai virus-induced clones under selection *in vivo* is determined by inheritable properties that are stable even after 15 months of culture *in vitro* or after HPRT deficiency.

The emergence of highly malignant tumors is accompanied by loss of chromosomes

The tumorigenicity of HS-5-1 was stable over 4 months *in vivo* (Fig. 1A) and there was no significant change in chromosome number (Fig. 1B). The increased tumorigenicity of NA-1 and SF-74A was associated with a decrease in the chromosome number (Fig. 1A and 1B). The Sendai virus-induced HVJ-2 line became more malignant in a biphasic manner (Fig. 1A). There is a good correlation between increased tumorigenicity and decrease of chromosome number of various variants except HS-5-1 during growth *in vivo* (Fig. 3).

Comparison of chromosome constitution of SF-74A with its malignant tumor after growth in vivo

The SF-74A line was selected to perform the banded chromosome analysis of the initial variants

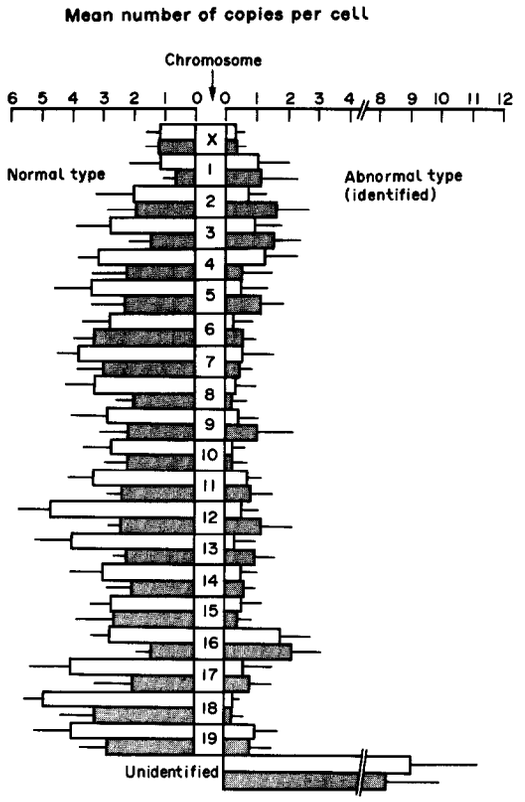

Fig. 6. Comparison of cloned SF-74A line and its ascites tumors obtained from the injection of 100 *cells at the 4th serial passage with respect to their chromosome composition.* □; *Mean number of copies per cell of chromosomes of SF-74A clone,* ■; *mean numbers of copies per cell of chromosomes of its tumors; vertical line in each bar, S.D.*

and the malignant tumors obtained after repeated passages. The SF-74A clone had a log TFD_{50} value of 4.8 and 84 chromosomes, whereas the ascites tumors had 69 chromosomes (Fig. 1). This indicated that the ascites tumors originating from the SF-74A clone eliminated an average of 15 chromosomes during growth *in vivo*. The karyotypes of the cloned SF-74A line and its ascites tumors obained from the injection of 100 cells at the 4th serial passage (after 71 days of maintenance *in vivo*) were compared with the use of the trypsin-Giemsa technique. The analysis of chromosome banding pattern was carried out on 20 metaphase preparations of both cells. A representative karyotype of SF-74A and the ascites tumor is shown in Figs 4 and 5.

The average number of copies of each chromosome of normal and abnormal origin in both cells is shown in Fig. 6. Compared with the average number (3.1) of copies of each normal chromosome in the SF-74A clone, the tumors had an average copy number of 2.1, one fewer than that of the initial clone. The tumors showed a decrease in a copy of chromosomes 3, 4, 5, 8, 14, 16, 18 and 19, and in 2 copies of chromosomes 12, 13 and 17. The average number of marker chromosomes of identified origin was 0.7 and 0.8 in the initial clone and the tumors, respectively. The average number of unidentified chromosomes in both cells is also presented in Fig. 6. Fifty-nine different markers and 20 different unidentified chromosomes were present in the 20 SF-74A clone, whereas 62 markers and 32 unidentified chromosomes were seen in the tumors. Twenty-three markers and 11 unidentified chromosomes from the SF-74A were also present in the tumors. There was no marker occurring in all analyzed tumor cells. Marker* and marker** in the tumors, shown in Fig. 5, occurred at a rate of 80 and 70%. The frequencies of the other markers were less than 40%. Thus, it was very difficult to find a correlation between the loss or gain of a specific chromosome with the increased tumorigenicity.

DISCUSSION

The EAT variants we have isolated are less tumorigenic and have more chromosomes than the original EAT. Many of these variant clones generate highly malignant tumors in a relatively short time when they are exposed to strong selective pressures in the mouse. The same variant clones maintained under non-selective growth conditions *in vitro* showed no tendency to generate malignant tumors over a similar time (Table 1). When these variants were passaged continuously *in vivo*, they showed 3 different patterns in their tumor progression (Fig. 1): (i) the tumorigenicity was unaltered and the loss of chromosomes was few as shown by the HS-5-1 clone which is markedly different from the other variants in chromosome number and composition, as well as the malignant phenotypes (manuscript in preparation); (ii) a rapid increase in malignancy, thus eliminating most of the less tumorigenic variants as shown by the NA-1, SF-74A and 1.2S-B lines with which a significant decrease of the chromosome number is always associated with an increase of tumor-forming ability; and (iii) a rapid increase in tumorigenic potential to cells that must be injected at 500 cells/mouse or greater to produce tumors as seen with the HVJ-2 and HVJ-X clones. This pattern was the same in the long cultured clone and enzyme-defective line of Sendai virus-induced variants (Fig. 2). The cytogenetic analysis of the clones and their tumors after growth *in vivo* pointed strongly to the importance of chromosomal changes in the appearance of tumor-forming ability. The banding analysis of chromosomes in the SF-74A clone and its tumors after 4 passages *in vivo* demonstrated that the majority of chromosomes segregated from the clone were of normal type, suggesting that the eliminated chromosomes were associated with the suppression of malignancy, although the association of the loss of a specific chromosome(s) with the suppression of malignancy could not be made. A similar correlation of decreased chromosome number with increased tumorigenicity has been shown in cell hybrid where there is a decrease in chromosome number from the teraploid range in

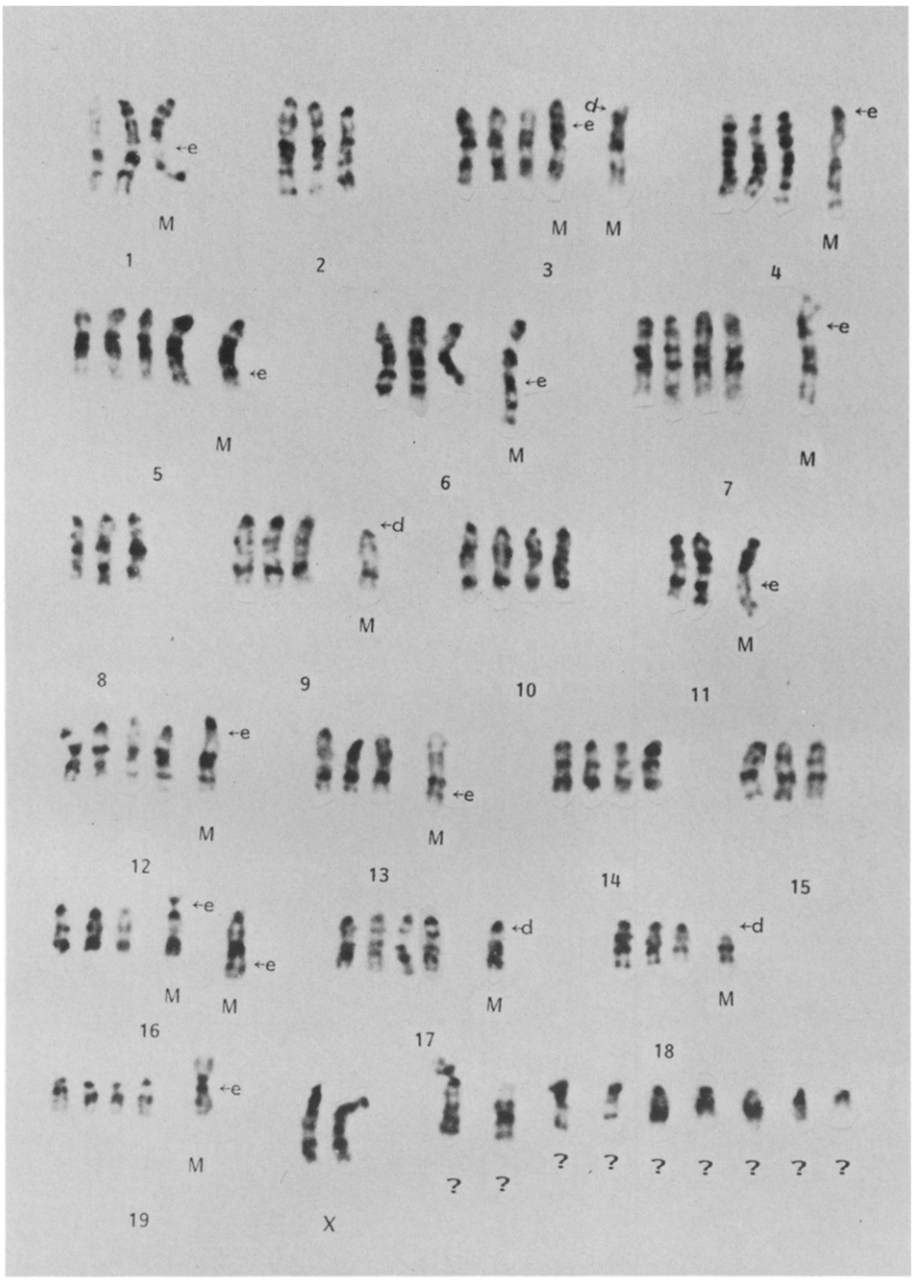

Fig. 4. Example of G-banded karyotype of a SF-74A clone. M, Marker chromosome; ?, unidentified chromosome; d, deletion; e, extra material.

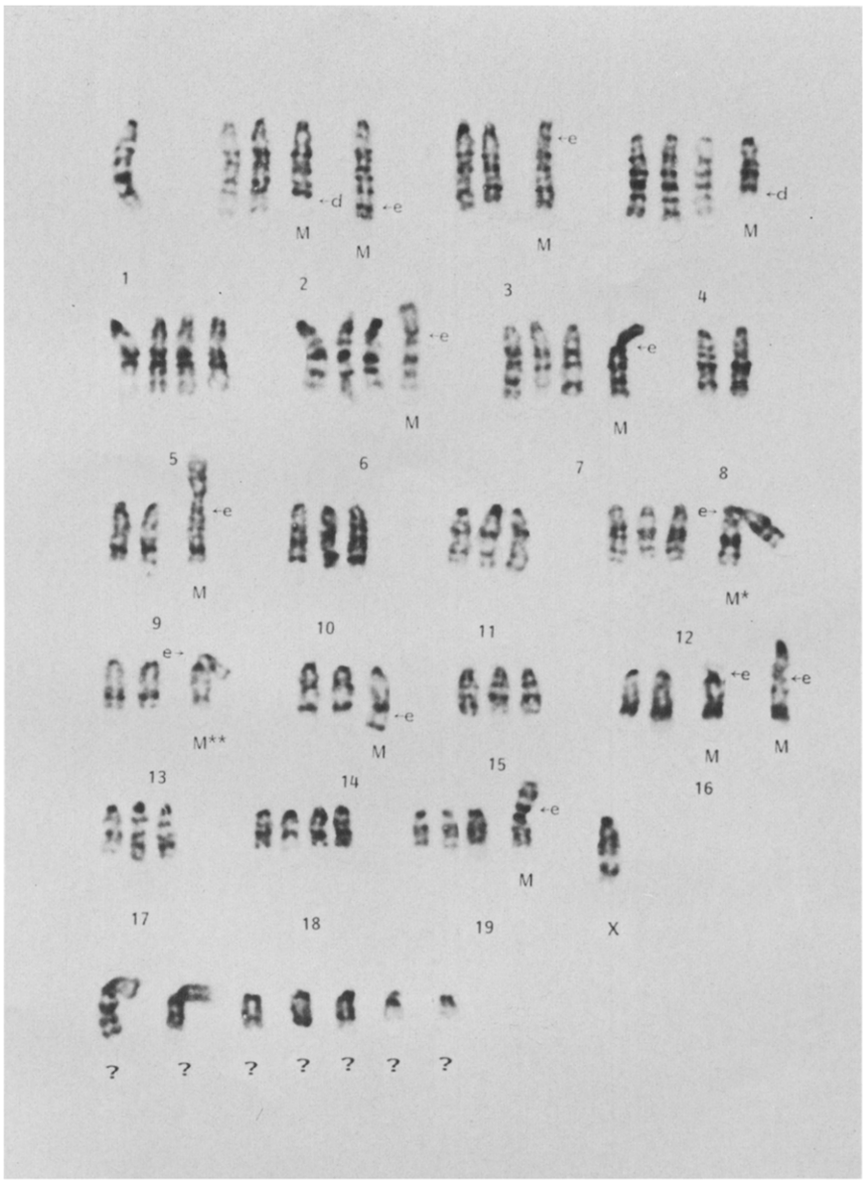

Fig. 5. Example of G-banded karyotype of a SF-74A in the ascites tumors derived from the injection of 100 *cells at the 4th passage* in vivo. *M, Marker chromosome*; ?, *unidentified chromosome*; *d, deletion*; *e, extra material. M*, rob t*(12:12); *M**, rob t*(?:13).

the initial hybrids to the diploid range in the tumors [12–14]. In mouse intraspecific hybrids, tumor suppression requires the activity of some gene located on the normal chromosome 4 [15 16]. In other studies, however, the loss of a combination of several chromosomes from the hybrids results in the re-emergence of tumor-forming ability that was initially suppressed [17, 18]. In our system, the suppression of the tumorigenicity was not associated with additional copies of a specific normal chromosome including chromosome 4. The loss of a combination of some normal chromosomes appeared to be important in the phenotypic transition. The same process of chromosome rearrangement that generates oncogene activity [19] may also be considered as a source of suppressor loss. However, there was no consistent gain of a single marker chromosome in the tumors in our experiment.

In our culture system, 3 variant types had an ability to induce rapid emergence of malignant tumors during growth *in vivo*. Such results are consistent with the current concepts that tumor progression mutation-like events occur spontaneously in tumors. Individual clones isolated from the heterogenous parental lines results in the formation of variants with increased malignancy and metastatic potential, in combination with the selectivity of the host environment [3, 4, 6].

In our experiment, new malignant tumors were generated and selected for their enhanced capacity to grow in the peritoneal cavity of normal recipients. But the rapid emergence of malignant tumors was not confined to such host pressures. There was a tendency to depend on the inheritable properties of the tumor cells themselves, as revealed by the differences in tumor progression of different variant clones during growth *in vivo*. The possibility that, before growth *in vivo*, the starting clone contained undetected variants which subsequently underwent selection *in vivo* is unlikely because variants capable of inducing malignant tumors were obtained from 3 independently isolated clones of different origin. From our experiment, the following conceptual framework has been shown that during growth *in vivo* EAT yielded less tumorigenic variants with more chromosome ([8], manuscript in preparation) and that the variant clones produced highly malignant tumors with the loss of chromosomes. We believe that this dynamic model can be employed for a better understanding of tumor heterogeneity and clonal instability of tumors.

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