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LEAD ARTICLE

Aneuploidy Precedes and Segregates with Chemical Carcinogenesis

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ABSTRACT: *A century ago, Boveri proposed that cancer is caused by aneuploidy, an abnormal balance of chromosomes, because aneuploidy correlates with cancer and because experimental aneuploidy generates "pathological" phenotypes. Half a century later, when cancers were found to be nonclonal for aneuploidy, but clonal for somatic gene mutations, this hypothesis was abandoned. As a result, aneuploidy is now generally viewed as a consequence, and mutated genes as a cause of cancer. However, we have recently proposed a two-stage mechanism of carcinogenesis that resolves the discrepancy between clonal mutation and nonclonal karyotypes. The proposal is as follows: in stage 1, a carcinogen "initiates" carcinogenesis by generating a preneoplastic aneuploidy; in stage 2, aneuploidy causes asymmetric mitosis because it biases balance-sensitive spindle and chromosomal proteins and alters centrosomes both numerically and structurally (in proportion to the degree of aneuploidy). Therefore, the karyotype of an initiated cell evolves autocatalytically, generating ever-new chromosome combinations, including neoplastic ones. Accordingly, the heterogeneous karyotypes of "clonal" cancers are an inevitable consequence of the karyotypic instability of aneuploid cells. The notorious long latent periods, of months to decades, from carcinogen to carcinogenesis, would reflect the low probability of evolving by chance karyotypes that compete favorably with normal cells, in principle analagous to natural evolution. Here, we have confirmed experimentally five predictions of the aneuploidy hypothesis: (1) the carcinogens dimethylbenzanthracene and cytosine arabinoside induced aneuploidy in a fraction of treated Chinese hamster embryo cells; (2) aneuploidy preceded malignant transformation; (3) transformation of carcinogen-treated cells occurred only months after carcinogen treatment, i.e., autocatalytically; (4) preneoplastic aneuploidy segregated with malignant transformation in vitro and with 14 of 14 tumors in animals; and (5) karyotypes of tumors were heterogeneous. We conclude that, with the carcinogens studied, aneuploidy precedes cancer and is necessary for carcinogenesis.*

INTRODUCTION

Over a century ago, asymmetric mitoses, which generate an abnormal balance of chromosomes or aneuploidy, were first discovered in epithelial cancer cells by Hansemann [1]. At about the same time, aneuploidy was shown experimentally to cause "pathological, lethal, and tumor-like" phenotypes in developing sea urchin embryos by Boveri [2]. On this basis, aneuploidy was proposed to cause cancer originally by Hansemann [1] and Boveri [2, 3] and then by others up to the 1960s [4–7].

Since the 1960s, however, the aneuploidy-cancer hypothesis has been abandoned by many cancer researchers in favor of the somatic gene mutation hypothesis, primarily because the cells of virtually all cancers were found to be highly heterogeneous, i.e. nonclonal, with regard to aneuploidy [8–13]. In the meantime, many cancers were found to be clonal with regard to one of many kinds of somatic gene mutations [14–18], including those caused by reciprocal chromosome translocations [19–21]. In view of the clonality of the gene mutations [14, 15], the nonclonal

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aneuploidies were interpreted as consequences of malignant transformation [10, 11, 13]. For example, Nowell wrote in an influential article in 1976, "It is certainly clear that visible alterations in chromosome structure are not essential to the initial change" [22]. And Cairns wrote in 1981, "changes in karyotype could . . . be trivial secondary events that occur after all the rate limiting steps of carcinogenesis have been completed" [23]. According to a prominent cancer textbook, "The dilemma is whether or not the karyotypic changes are the result or the primary cause of neoplasia" [15]. In a recent issue of *Science*, one of two articles comments that it is "still unresolved . . . whether an increase in ploidy contributes to, or is a consequence of, tumor development" [24], and the other states that "hyperploidy in tumor cells is usually viewed as a consequence . . ." [25].

However, despite enormous efforts, there is as yet no evidence that the gene mutations of any cancers are cancer-specific [18, 26–31], and there is as yet no functional proof that one or a combination of mutated genes from cancer cells can transform diploid human or animal cells to cancer cells [26, 31–39]. And the failure of mutated genes from cancer cells to transform diploid cells is not just a technical problem, because chromosomally integrated retroviral transforming genes from cancer cells have transforming function [35, 37, 40–42]. In view of this, we [43–45] and others [46] have recently reconsidered all evidence for and against the aneuploidy hypothesis.

Since the times of Hansemann and Boveri, aneuploidy has continued to be the most common and massive genetic abnormality of solid cancers to this day [12, 47, 48]; even the chromosomally encoded centrosomes of all solid cancers tested were recently shown to be structurally and numerically altered [46, 49–51]. According to Oshimura and Barrett, "a better correlation with cell transformation is observed with induction of aneuploidy than point mutations" [52]. Moreover, the ability of aneuploidy to mutate eukaryotic phenotypes originally demonstrated by Boveri has recently been confirmed experimentally in plants [54], yeast [55], and Drosophila [56], and descriptively by noncancerous aneuploidies in humans [57–59]. At the same time, aneuploidy and its corresponding phenotypes continue to be nonclonal in cancers [6, 13, 29, 44, 53] that are clonal for certain, albeit unspecific gene mutations (see above).

In view of this, and in an effort to reconcile the nonclonal karyotypes with the aneuploidy-cancer hypothesis, we arrived at the following two-stage mechanism of carcinogenesis (see Fig. 1):

1. In stage 1, a carcinogen "initiates" [14, 15] carcinogenesis by generating a preneoplastic aneuploidy. For this purpose, the carcinogen must function as an aneuploidizing agent—for example, by disabling mitosis proteins either physically or chemically [52, 60–62]. Indeed, 99% of the best chemical carcinogens, the polycyclic aromatic hydrocarbons [63], bind to or react with proteins instead of nucleic acid [64].
2. In stage two, aneuploidy propagates and varies itself, because it destabilizes the karyotype—a process that has been termed "chromosome error propagation" [65]. The mechanism is that aneuploidy biases balance-sensitive spindle and chromosomal proteins [55, 66, 67], and alters the composition and even number of centrosomes (see above). Therefore, the karyotype of an initiated cell evolves autocatalytically, generating randomly ever-new, abnormal karyotypes including lethal, preneoplastic, and neoplastic ones [43–45, 65] (Fig. 1). The degree of karyotypic instability would be proportional to the degree of aneuploidy [44]. The heterogeneous karyotypes and phenotypes of "clonal" cancers would thus be an inevitable consequence of the intrinsic instability of aneuploid karyotypes. Cancers would be "clonal" for aneuploidy, but not for the karyotypes of individual cells.

Because the probability to evolve by chance a karyotype that grows better than a normal cell is very low, the evolution of a neoplastic karyotype is very rare and thus typically a late outcome of autocatalytic karyotype evolution. Indeed, the majority of random karyotypes would be lethal, but those with preneoplastic and neoplastic phenotypes would survive [3, 6, 68, 69] (Fig. 1). In the words of Boveri, the odds for a neoplastic karyotype are "as low as winning in a lottery" [3]. The notorious long latent periods from carcinogen exposure to carcinogenesis, i.e., months to decades [8, 9, 14, 15, 70], confirm this view.

Thus our hypothesis predicts an as-yet poorly defined threshold for neoplastic aneuploidy [45] (Fig. 1). Aneuploidy below this threshold would not be cancerous and would involve few and predominantly small chromosomes. Examples include Down syndrome with a trisomy or monosomy of chromosome 21 [12] and the preneoplastic aneuploidies that are postulated to cause hyperplasia, dysplasia, and immortalization of cells in vitro (see below).

The effects of aneuploidy on the phenotype of the cell are analogous to those of randomizing assembly lines of a car factory, i.e., cars with abnormal ratios of normal (rather than mutated) wheels, bodies, and engines. In other words, the effects of both positive and negative aneuploidy are always dominant [45, 56–59, 71–73], whereas the effects of gene mutations are typically recessive [14, 71, 72, 74]. Accordingly, nature uses gene mutation for minor adjustments within a species, but reserves chromosome number mutations for major irreversible changes such as the generation of new species.

Here we describe experiments testing five specific predictions of the aneuploidy-cancer hypothesis: (1) carcinogens, such as dimethylbenzanthracene (DMBA), methylcholanthrene (MCA), and cytosine arabinoside (ara-C), induce aneuploidy in Chinese hamster embryo (CHE) cells; (2) aneuploidy precedes malignant transformation; (3) transformation of initiated cells is slow (compared to mutation) and carcinogen-independent; (4) aneuploidy segregates with malignant transformation in vitro and with tumors in animals; and (5) the karyotypes of clones of cells transformed in vitro and of tumors in animals are heterogeneous. As in a previous study [43], DMBA and MCA were also used here because these carcinogens transform, but do not detectably mutate CHE cells in culture

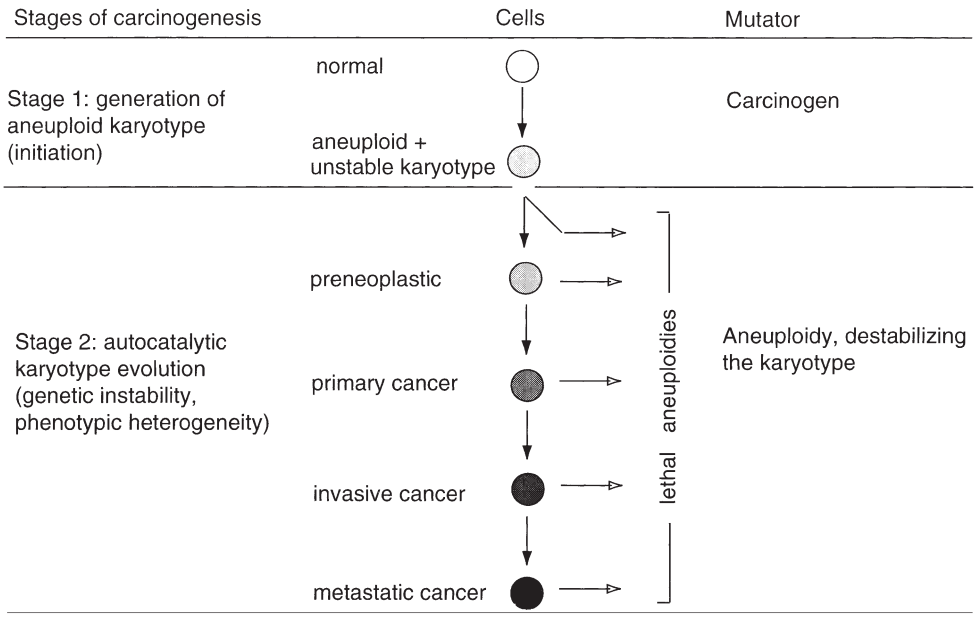


Figure 1 A two-stage model for how carcinogens may cause cancer via aneuploidy. In the first stage, a carcinogen "initiates" carcinogenesis by generating an aneuploid, preneoplastic cell. Because aneuploidy destabilizes the karyotype, through unbalancing spindle proteins by unbalancing their chromosomal templates (see text), the aneuploid preneoplastic cell will autocatalytically generate new karyotypes, including those of preneoplastic and neoplastic cells. The autocatalytic karyotype evolution would explain the previously unresolved, carcinogen-independent transformation of a preneoplastic into a neoplastic cell. The notorious long latent periods from initiation to carcinogenesis would be a consequence of the low probability of generating by chance a karyotype that can outperform normal cells. The same process will generate more frequently nonviable chromosome combinations (i.e., cell death) (see text).

[61, 62]. Thus, as nongenotoxic carcinogens in this system, they are expected to transform via aneuploidy. By contrast, ara-C was studied as a genotoxic carcinogen that may transform by mutation without generating aneuploidy.

MATERIALS AND METHODS

Cell Culture

CHE cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum and 5% calf serum, an antibiotic/antimycotic mix (GIBCO/BRL), 50 μg per mL gentamycin, and 0.2% mycostatin (GIBCO/BRL) following published procedures [43]. CHE cells were treated with 1 μM DMBA in medium containing 0.1% DMSO, and with 1 μM ara-C in medium without DMSO. Control cells were treated with the same medium as experimental cultures. Media were changed every other day.

Chromosome Analysis

Subconfluent cultures of chemically-treated or untreated CHE cells that had been kept growing for one or two consecutive passages in culture were incubated for 2–3 hours with 0.6 μg/mL of Colcemid (Gibco/BRL). The cells were then rinsed with phosphate-buffered physiological saline, dissociated with trypsin at 37°C, mixed with 1 mL complete culture medium, and centrifuged for 6 minutes at 500 rpm at room temperature. Subsequently, the cells were resuspended at room temperature in 250 μl of the above mixture and 6 mL of 75 mM KCl (Gibco/BRL), and incubated for 12–20 minutes. The cells were then fixed by mixing the solution with an equal volume (6 ml) of ethanol/acetic acid (3:1, v/v) and incubated for 30 minutes at room temperature. After centrifugation (as above) the cells were resuspended in 10 mL of ethanol/acetic acid (3:1, v/v) and incubated for 10–15 minutes and again centrifuged. The cells were then resuspended in 0.25–0.5 mL of the same solvent and dripped on a tilted (45 degrees) microscope slides from about 1-cm height (3 drops per slide, side-by-side). Metaphase chromosomes were counted with a phase-contrast microscope at 400× and 630× magnification, either directly after air drying or after incubation at 55°C overnight with or without Giemsa staining.

Chemical Carcinogenesis

For carcinogenesis, 3–6-month-old inbred male Chinese hamsters [80, 85] were injected intramuscularly in the upper thigh with 1 mg DMBA or 1 mg MCA in 0.1 mL tricaprylin (Sigma), as described previously [75, 76]. Animals were monitored for tumor formation by inspection and palpation at weekly intervals. Tumors of 1–1.5-cm diameter were excised from euthanized animals. For karyotype analysis, tumors were minced with two scalpels, washed with physiological saline, and trypsinized while stirring

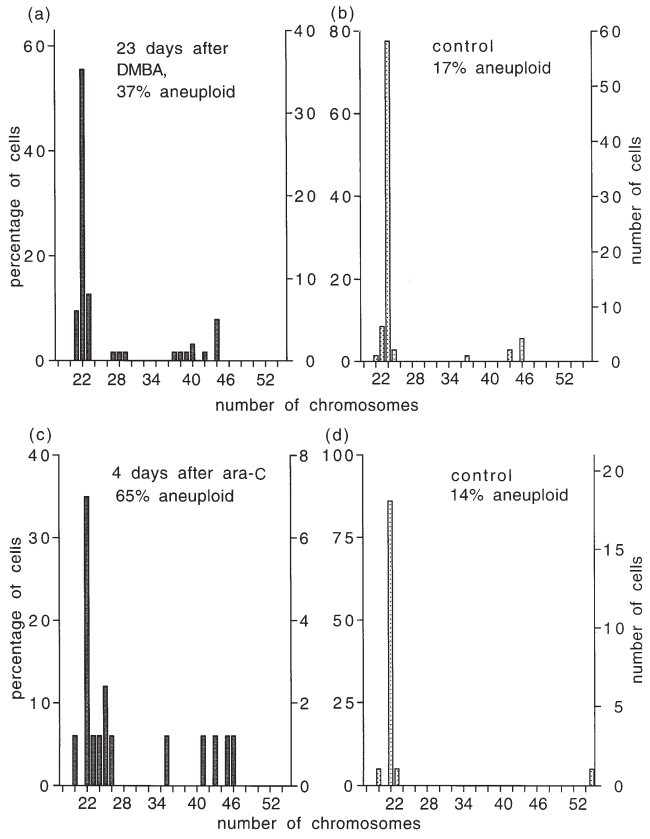
at 37°C. Cells were then cultured to confluency, and then retrypsinized for karyotype analysis, as described above.

RESULTS AND DISCUSSION

Carcinogens Induce Aneuploidy Prior to Transformation

To test the prediction that carcinogens can function as aneuploidogens (Fig. 1), we have analyzed the chromosomes of CHE cells after treatment with a transforming concentration of DMBA, but prior to transformation. Because malignant transformation takes at least 2 months under these conditions [43], we have analyzed the karyotypes of CHE cells 23 days after treatment with this carcinogen. It is shown in Figure 2a that 37% of CHE cells were aneuploid 23 days after initiation of treatment with DMBA, compared to 17% of untreated controls (Fig. 2b). The DMBA-induced aneuploidy fell mostly in the hyperdiploid range. This is the same numerical range of chromosomes as that of CHE cells that had been malignantly transformed with DMBA in previous studies [43, 44].

Figure 2 The chromosome distribution of Chinese hamster embryo (CHE) cells 23 days after treatment with cell-transforming concentrations (1 μM) of dimethylbenzanthracene (DMBA) (a), and 4 days after treatment with cell-transforming concentrations (1 μM) of cytosine arabinoside (ara-C) (c). The chromosome distribution of untreated control cells, after 6–12 population doublings in cell culture, is shown in (b) and (d). The data show that, after subtracting the background levels of the controls (see text), about 20% of CHE cells were rendered aneuploid by DMBA, and 51% by ara-C.



Transformation of CHE cells with ara-C, which is carcinogenic for rodent cells at 1 μM [77, 78], also occurs only about 2 months after initiation of treatment (unpublished). It is shown in Figure 2c that 65% of CHE cells were aneuploid 4 days after initiation of treatment with 1 μM ara-C, compared to 14% of untreated controls (Fig. 2d). As was the case with DMBA, the ara-C-induced aneuploidy fell mostly in the hyperdiploid range. The high percentage of aneuploidy observed soon after ara-C treatment appears to be unstable, as the percentage of aneuploidy declines on further passage (unpublished). High initial rates of aneuploidy shortly after carcinogen treatment have been described by others and attributed to the non-viability of most randomly altered karyotypes [79].

According to the literature the relatively high percentage of aneuploidy (14–17%) of untreated CHE cells, three to five passages in cell culture after preparation from the embryo, is due to artifacts of two kinds: (1) losses, and rarely, gains of metaphase chromosomes from spreading hypotonic nuclei for karyotype analysis; and (2) spontaneous aneuploidization that occurs in cell culture, although the cells of normal Chinese hamsters are diploid [6, 80–82]. Thus, after subtracting the background of untreated controls, approximately 20% of CHE cells were rendered aneuploid by DMBA, and 51% by ara-C.

We draw two conclusions: First, the two carcinogens tested function as aneuploidogens. The rather high yields of aneuploidy prior to transformation are compatible with the hypothesis that the carcinogens interfere directly with mitosis, and virtually exclude the hypothesis that, under our conditions, the aneuploidy was caused by carcinogen-mediated mutation of mitosis genes. This is particularly true for DMBA, which is not even mutagenic in cultured CHE cells [61, 62]. Second, aneuploidy precedes transformation of CHE cells. This is kinetic evidence for a cause, rather than a consequence of transformation. Nevertheless, it could be argued that aneuploidization is a transformation-independent event, that is not necessary for transformation.

In Carcinogen-treated Cells, Aneuploidy Segregates with Malignant Transformation In Vitro and In Vivo

Next, we have used a statistical argument to determine whether aneuploidy is necessary for malignant transformation, or is a transformation-independent event. If aneuploidy were necessary for transformation, one would expect that, in a population of carcinogen-treated cells, preneoplastic aneuploidy would segregate with malignant transformation. In other words, all transformants would be aneuploid, even though only a fraction of the carcinogen-treated cells are. If aneuploidy were not necessary for transformation, the fraction of aneuploid transformants would be the same as the fraction of aneuploid cells in the carcinogen-treated, preneoplastic precursor cells.

In a previous analysis, we have already found that all 38 of 38 transformed colonies arising from CHE cells treated in vitro with the polycyclic aromatic hydrocarbons, DMBA, MCA, and benzopyrene, were aneuploid [43]. Because the percentage of aneuploid CHE cells in

polycyclic aromatic hydrocarbon-treated cultures prior to transformation ranges from 20% (Fig. 2) to a maximum of 46% [80], the odds that all 38 transformed colonies are aneuploid would at most be $0.46^{38} = 10^{-13}$ or practically zero. This estimate assumes the highest preneoplastic rate of aneuploidy (i.e., 46%) that has been reported. This suggests that aneuploidy is necessary for transformation [43].

However, it has been demonstrated that spontaneous aneuploidization of CHE and other rodent cells propagated in cell culture is high [5, 15, 80, 82]. Therefore, it could be argued that aneuploidy confers a growth advantage to cells in culture, and that the perfect correlation between transformation and aneuploidy in vitro reflects a selection for growth in culture rather than a condition for malignant transformation. In view of this, we have analyzed the chromosomes of tumors induced with DMBA and MCA in young (3–6-months old) adult Chinese hamsters (Materials and Methods). Because spontaneous aneuploidy in animals, particularly young animals, is negligible [6, 80], and only a minority of cells treated for a short time with DMBA or MCA are rendered aneuploid, the majority of DMBA and MCA-induced tumors should be diploid, if aneuploidy were not necessary for tumorigenesis.

The tumors were induced by a one-time inoculation of 1 mg DMBA or MCA in the thigh of the hamsters, as described in Materials and Methods. Fourteen of 23 injected animals developed tumors 3–9 months later at the site of inoculation, after an average latent period of 6 months (Table 1). To determine the karyotypes, the tumors were excised and dissociated into single cells with trypsin. The tumor cells were then plated onto petri dishes and the metaphase chromosomes of Colcemid-treated cultures were determined within a few days after explanting the tumors (Materials and Methods).

As shown in Table 1, all 14 tumors were aneuploid. The percentage of aneuploidy varied from 50% to 97%. The presence of diploid cells in our tumor preparations is expected because all tumors contain normal stromal supply tissue [13], and because surgical preparations often include nontumor tissue as well. The real percentages of aneuploidy are probably higher because we have not identified nor subtracted pseudodiploid cells from the diploid counts of our tumors [80, 83]. Thus, all 14 tumors were aneuploid (Table 1). Nevertheless, the presence of some diploid tumor cells within aneuploid tumors can not be excluded without cloning tumor-derived diploid cells and testing them for tumorigenicity.

To estimate the probability that all 14 tumors arising from DMBA- and MCA-treated hamsters would be aneuploid by chance, one would have to know the percentage of cells rendered aneuploid prior to transformation by a one-time exposure to these carcinogens. However, the number of cells exposed to carcinogen in injected animals is difficult, if not impossible, to determine. Therefore, we assume that, in a first approximation, the percentage of aneuploidization in vivo by a one-time exposure to DMBA is the same as in vitro (i.e., 20%, see Fig. 2a). This is an upper limit, because a one-time treatment of the skin of mice with a transforming dose of polycyclic aromatic hydrocarbons has likewise rendered initially only a small percent-

Table 1 Distribution of karyotypes in individual tumors

Tumor	Incubation (month)	mn	Aneuploid %	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	Above/below
D-T1	5	40	92				1				1					1		2		1		1	1	10	2	1	2	3	2	6	1		1 × 58
D-T2	6	24	97	1	1	1		6	1	1			1					1	1	2		1	1	1	2	4		1	2	1			
D-T3	8	22	50	2	3	14	8		1																								
D-T4	8	22	56	2		11	1	8	1		1						1																
D-T6	3	41	93			1		1	1										1					1	1	3		2		1		1		1 × 51,60
D-T12	4	22	68	2	5	17	12	7	3				1						2					1		1			1	1	4		
D-T16	7	23	89	1		7	16	6	1					1					2	2	2	7	5	6	6	4		1	1	1			1 × 53
D-T21	6	22	52	3	19	43	17	1														1	2			6	1	7	4	1			
D-T22	5	23	73		2	12	15	4	2	1		1	1		2	1			2	1		1			3	1	3	1					1 × 52, 54
M-T1	7	22	77	2		8		1		5			1	1		2		2	2	5	1		1					1					1 × 16, 17, 19
M-T15	5	23	81	1		11	27	6	3	1		1							2				1				1						1 × 50
M-T18	9	25	89			1	4	7	4	8	5	2			1											1			1		1	1 × 50, 52
M-T19	9	25	91			1	6	11	12	20	10	3											1				1		2	1	1		1 × 53
M-T22	4	22–23	84	2	2	14	14	4	2	4		4			4			12	1	2	4	2	4		4		4						2 × 56
average	6																																

Addreviations: mn, modal chromosome number; D, DMBA-induced; M, MCA-induced tumors.

age (<10%) of treated cells aneuploid [84]. Skin carcinogenesis is a system in which, in contrast to ours, carcinogen-exposed cells are accessible in vivo. Using the value of 20%, the odds that all of the 14 tumors would have evolved from an aneuploid cell are only $0.2^{14} = 1.6 \times 10^{-10}$ or practically zero. Thus, aneuploidy must be necessary for tumorigenesis because it segregates very specifically with tumors induced by DMBA and MCA.

Tumorigenesis 3–9 Months After One-Time Treatment with Carcinogen

The latent periods from DMBA- and MCA-treatment to carcinogenesis in 14 hamsters ranged from 3 to 9 months, with an average of 6 months (Table 1).

Such long latent periods are incompatible with the mutation hypothesis. Mutation from a one-time treatment with carcinogen should occur almost instantly because enzymatically oxidized DMBA reacts with the DNA of the animal in less than 24 hours [122]. Assuming a mutational mechanism, the first tumor cell should have been generated in our conditions within a month after treatment, as most of the polycyclic hydrocarbons injected for tumorigenesis are excreted within a month [75]. If that tumor cell had the same generation time as normal CHE cells in culture (i.e., 14 hours) [85], it could generate within one month a tumor of about 3×10^{15} ($= 2^{30 \times 24/14}$) cells, the equivalent of 1,000 hamsters. Thus, a mutation-initiated tumor should appear within less than 2 months after treatment with carcinogen. The assumption that a tumor cell can grow approximately as fast as normal CHE cell is consistent with our experience with dozens of chemically transformed CHE cells, which grew as fast, if not faster than, normal cells [43, 44]. Because the latent periods of all tumors exceeded 2 months, they signal a non-mutational mechanism as predicted by the aneuploidy hypothesis.

Indeed, our results confirm two predictions of the aneuploidy hypothesis: (1) The tumors originate from "initiated" cells in the absence of carcinogen, that is, at a time when most or all inducing carcinogen has been excreted [75]. (2) The time from carcinogen treatment to carcinogenesis is long and very variable, because of the low probability that chromosome combinations would evolve by chance that are more viable than the normal karyotype—as anticipated by Boveri.

It may be argued that the first tumor cell originated from somatic mutation soon after carcinogen treatment, and that the long and different latent periods reflect extremely slow growth rates of the tumor cells. In this case, the generation time of the average tumor cell would be about 7 days (180/26) or about 12 times ($7 \times 24/14$) slower than that of a normal CHE cell (14 hours), because the average tumor appeared only 6 months (180 days) after initiation with carcinogen and consisted of about 10^8 cells, which corresponds to about 26 cell doublings of the founder cell. However, several arguments refute this hypothesis: (1) Once identified by palpation all tumors grew fast (i.e., within a few weeks) to 1–1.5 cm, when they were harvested for karyotype analysis. (2) The explanted cells of all tumors grew at about the same rates in vitro as normal cells or cells chemically transformed in vitro.

We conclude that the long and variable latent periods for tumorigenesis reflect the low probability of generating by chance the first tumorigenic cell from preneoplastic precursors. This agrees with our proposal, that the "epigenetic" event that has been postulated to explain the slow and carcinogen-independent evolution of neoplastic cells from preneoplastic precursors [101, 124], is autocatalytic karyotype evolution (see below).

Karyotypes of DMBA- and MCA-induced Tumors Are Heterogeneous

It is also shown in Table 1 that the karyotypes of the cells of each of the 14 tumors were heterogeneous, and that the modal chromosome numbers were either near diploid or near tetraploid. This confirms others who have also demonstrated heterogeneous karyotypes in chemically-induced cancers [86–89]. This karyotypic heterogeneity is consistent with the hypothesis that aneuploidy destabilizes the karyotype and thus generates tumors that are clonal for aneuploidy but not for the karyotypes of individual cells.

CONCLUSIONS

Necessity of Aneuploidy for Cancer

Our experiments have confirmed five predictions of the aneuploidy-cancer hypothesis: (1) the carcinogens tested functioned as aneuploidogens; (2) aneuploidy preceded malignant transformation; (3) transformation of initiated cells was slow, compared to mutation, and carcinogen-independent, i.e., autocatalytic; (4) aneuploidy among carcinogen-treated cells segregated with malignant transformation; and (5) the karyotypes of all tumors were heterogeneous.

Unexpectedly, in view of the currently prevailing somatic mutation hypothesis [17, 90], there is support for most of our observations hidden in the huge literature on cancer. For example, several researchers have demonstrated that carcinogens function as aneuploidogens [52, 60, 62, 91, 92]. Others have observed aneuploidy prior to chemical transformation in vivo [84, 93–95], in vitro [69, 96–101], and prior to spontaneous transformation in vitro [5, 82, 102]. Indeed, preneoplastic aneuploidy of human biopsies has been studied as an indicator of the cancer risk [103–110]. But these observations have probably failed to make a decisive impact on the question of whether aneuploidy is a cause or consequence of cancer, because aneuploidy was studied either as a cofactor of, or as a source of somatic gene mutations, rather than as an independent cause of cancer. For example, Vogelstein et al. have recently postulated that aneuploidy "drives tumor progression by generating mutations in oncogenes and tumor-suppressor genes", but the question of how aneuploidy would mutate genes, other than by altering their dosage, was not answered [111].

We conclude that, with the carcinogens tested, aneuploidy is not a consequence of transformation. Instead, it precedes cancer and is necessary for carcinogenesis.

Aneuploidy versus Gene Mutation as an Explanation of Cancer

In this section we compare the abilities of the aneuploidy and gene mutation hypotheses to explain the complex

cancer-specific phenotypes and genotypes, as well as the exceedingly slow kinetics from carcinogens to carcinogenesis (see above and Table 2).

Cancer. The list of cancer-specific phenotypes includes abnormal, cellular and nuclear morphology, metabolism, growth, abnormal DNA indices ranging from 0.5 to >2, abnormal centrosome numbers, dedifferentiation or anaplasia, invasiveness, metastasis, and neoantigens (see above) [9, 14, 15], as well as "genetic instability" of all of these phenotypes and the corresponding heterogeneity of these phenotypes within individual cancers [6, 22, 29, 44]. As the following examples show, this list is more compatible with aneuploidy altering the dosage of thousands of regulatory and structural genes than with gene mutations (see Table 2):

1. The aneuploidy hypothesis predicts the abnormal DNA indices, from 0.5 to >2, i.e., aneuploidy, and therefore the abnormal nuclear and cellular morphology of cancer cells [8, 15]. By contrast, the mutation hypothesis predicts cancers that are diploid, just like conventional mutations; however, diploid cancers are virtually never described (see above). To reconcile aneuploidy with mutation, the mutation hypothesis would have to demonstrate that most of the known, hypothetical oncogenes are also aneuploidy-inducing genes, but this has not been done.
2. Aneuploidy predicts dedifferentiation or anaplasia, neoantigens, invasiveness, and metastasis [6, 15] because it regroups thousands of regulatory and structural genes, a process roughly similar to speciation. Since a species is defined by a specific number of chromosomes [112], aneuploid cancers must be viewed as species of their own, albeit parasitic ones. By contrast, mutation of one or a few genes is unlikely to inactivate, and even less likely to activate, a sufficient number of genes to generate these complex phenotypes [45, 72–74].
3. Aneuploidy predicts the abnormal structures and numbers of centrosomes in cancer cells because of the abnormal copy numbers of the corresponding chromosomal templates [51]. The mutation hypothesis does not offer an explanation for these abnormalities.
4. The aneuploidy hypothesis predicts the massive positive and negative shifts in the transcription of large numbers of genes that are typical of cancer cells [32, 113]. By contrast, either no or only modest shifts in transcription are predicted by one or several somatic mutations, particularly since most of the clonal mutations of cancer cells are also observed in noncancerous cells and in transgenic and spontaneously mutated noncancerous animals [114–118] (see also [35, 43] for examples).
5. Positive and negative shifts in growth rate corresponding to biases in regulatory and metabolic genes. This is totally consistent with aneuploidy, and at least partially with gene mutations.
6. The aneuploidy hypothesis explains why cancer-specific phenotypes are nonclonal and unstable, i.e., the genetic instability of cancer cells and the resulting heterogeneity of individual cancer cells (see above). By contrast, the mutation hypothesis predicts stable and specific cancer phenotypes, as for conventional mutations. According to the mutation hypothesis the phenotypes of cancer cells should be at least as stable and clonal as the presumably causative gene mutations of cancer cells, but this is not observed.
7. The consistent failure to find in cancer cells "dominant" [17, 90] transforming genes, capable of transforming diploid cells into cancer cells (see above), is compatible with the aneuploidy hypothesis, but not with the mutation hypothesis.

Carcinogenesis. The outstanding unexplained properties of carcinogenesis are the exceedingly long latent periods,

Table 2 Cancer and carcinogenesis as predicted by the aneuploidy and gene mutation-cancer hypotheses

Cancer	Aneuploidy	Mutation
1. Abnormal nuclear and cellular morphology corresponding to abnormal DNA indices, from 0.5 to >2, and aneuploidy	Yes	No
2. Anaplasia or dedifferentiation generating invasiveness, metastasis, and neoantigens	Yes	No
3. Abnormal centrosome structures and numbers	Yes	No
4. Massive positive and negative shifts in transcription	Yes	No
5. Positive and negative shifts in growth rate	Yes	Maybe
6. Genetic instability resulting in phenotypic heterogeneity of cells from individual cancers	Yes	No
7. Dominant transforming genes	No	Yes
Carcinogenesis		
1. Non-genotoxic carcinogens and tumor promoters	Yes	No
2. Latent periods of months to decades from carcinogen to cancer	Yes	No
3. 1000-fold age bias of cancer	Yes	No
4. Progression of malignancy	Yes	No
5. Cancer-normal cell hybrids may be nontumorigenic, but regain tumorigenicity by chromosome loss	Yes	Yes

of months to decades, from carcinogen to cancer, the 1000-fold age bias of cancer (see above), and the notorious progression of malignancy of in situ cancers to invasive and metastatic variants [9, 14, 15, 70, 119]. In addition, it is unexplained by the mutation hypothesis how nongenotoxic carcinogens cause cancer (see above).

1. The aneuploidy hypothesis predicts the growing lists of nongenotoxic carcinogens that are incompatible with the mutation hypothesis [33, 52, 62, 120, 121]. It also predicts non-genotoxic tumor promoters [15]. The nongenotoxic carcinogens and promoters are thought to function via aneuploidy, by physical or chemical reactions with the spindle or chromosomal proteins (see above).
2. The aneuploidy hypothesis predicts the exceedingly long and unpredictable latent periods between carcinogen treatment and cancer by autocatalytic karyotype evolution (Fig.1). By contrast, the mutation hypothesis predicts malignant transformation of a cell to coincide with carcinogen treatment, i.e., to be as fast as the reactions between a given carcinogen and DNA, as in conventional mutation. For example, polycyclic hydrocarbons react with proteins and DNA of mouse skin in less than 24 h [122], but cancer occurs on average only 6 months later [14, 15] (see also Table 1). In view of this, an "indirect" [123], or "epigenetic" [101, 124], or "unusual genetic event" was postulated "that cannot be the direct result of the lesions produced in DNA by the initial dose" of carcinogen [125]. According to Cairns: "the creation of a cancer cell is thought to involve a sequence of events of which perhaps only the early steps bear any direct relation to the interaction between mutagen and DNA" [23]. We propose that autocatalytic karyotype evolution is this "epigenetic sequence of events" that is rate-limiting. This interpretation of the long latent periods of carcinogen-induced carcinogenesis is supported by experimental carcinogenesis in which authentic human or animal cancer cells are transplanted into athymic mice or isogenic animals. Since there is no rate-limiting karyotype evolution in these systems, cancers appear within weeks, e.g., as fast as the implanted cells can grow [8, 15]. Thus the riddle, unexplained by the somatic gene mutation hypothesis, why a one-time treatment with carcinogens results in tumors only months or years later, long after the inducing carcinogen has reacted with components of the cell [14, 15], is explained by autocatalytic karyotype evolution.
3. Since aneuploidy is not heritable [126] and only slowly evolves chromosome combinations with neoplastic phenotypes, the aneuploidy hypothesis offers an explanation for the 1000-fold age bias of cancer [14, 15, 127]. However, the mutation hypothesis predicts cancer in newborns and predicts its incidence to increase linearly from birth, if one assumes a single mutation. Even if multiple mutations are postulated [18, 128, 129], cancer should also occur in newborns who have inherited all but one of a hypothetical complement of transforming mutations.
4. The notorious progression of malignancy of cancers in situ to invasive, metastatic, and drug-resistant variants [15] is also explained by autocatalytic karyotype variation and selection. By contrast, the mutation hypothesis would have to postulate mutations that are independent of those that generated the primary tumor, and would have to explain why such mutations are not commonly found to confer invasive, metastatic, and drug-resistant potential to otherwise normal cells.
5. The low probability of a neoplastic karyotype also explains why fusion of cancer cells with normal cells often, but not always, generates nontumorigenic cell hybrids [15]. Such fusions would destroy the rare neoplastic chromosome combination; however, such hybrids typically regain neoplastic properties by differential loss of chromosomes [15], driven by the karyotypic instability of aneuploid cells. However, assuming loss and recovery of tumor-suppressor genes, the mutation hypothesis can explain this as well.

In view of this and our data, we suggest that the aneuploidy-cancer hypothesis has unexplored potential to improve cancer prevention, by identifying and controlling aneuploidogens. One large European epidemiological study has already demonstrated that the degree of aneuploidy in lymphocytes accurately predicts an individual's cancer risk [130]. If confirmed, the hypothesis could also improve therapy, by distinguishing benign, and presumably diploid, from preneoplastic, and presumably aneuploid, lesions.

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