*MALIGNANT CONVERSION OF CELLS IN VITRO BY CARCINOGENS AND VIRUSES**

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Communicated by E. L. Tatum, June 21, 1966

Tumors may be regarded as arising from the conversion of one or, at most, a few normal cells. A permanent change, presumably in the cell genome, might result whenever cells are affected by a tumorigenic virus or chemical carcinogen. The work described in this paper is an attempt to observe such changes of cells *in vitro*, and to correlate what is seen with their ability to cause tumors when injected into animals. To reduce the morphological and karyological variability encountered with heterogeneous cell populations, such as those derived from trypsinized whole embryos, all experiments were begun with the same clone of a continuous line of cells which does not produce tumors. Colonies derived from single cells of this clone can easily be selected after growth in soft agar.[1] It thus became possible to examine the descendants of a single cell before and after treatment with carcinogens and viruses, and to determine what cellular changes were associated with the acquisition of an ability to grow into tumors *in vivo*.

Materials and Methods.—The cells used were obtained from Dr. T. C. Hsu in 1963. In 1964, this pseudodiploid, male Chinese hamster lung tissue cell line was cloned and adapted to grow as colonies in a semisolid agar medium as described previously[1, 2] Thereafter, routine passages in agar culture had a plating efficiency of approximately 10%. Traditional carcinogens were used: 7,12-dimethylbenzanthracene (DMBA), 3-methylcholanthrene (MCA), and 3,4-benzo(a)pyrene (BP). Fresh stock solutions in either dimethylsulfoxide (DMSO),[3] or dimethylformamide (DMF) (1–2 mg/ml), were added to the agar medium at the time the plates were poured, at an initial concentration of 0.3–0.5 μg/ml for the hydrocarbons and not above 0.2% for the solvents. Cells (*ca.* 10^6 in molten soft agar) were poured onto an agar underlay containing hydrocarbon. They were grown in the continuous presence of hydrocarbon for 12 days and then transferred to agar medium without carcinogen.

SV40 virus was obtained from Dr. J. Fogh, and a stock prepared using secondary cultures of green monkey kidney cells (titer of 5×10^8 PFU/ml[4]). A polyoma virus stock of similar titer (1×10^8 PFU/ml) was prepared on mouse embryo kidney cells as already described.[5] Virus stocks were kept frozen at −20°C. Tumor-inducing viruses were incubated overnight with 2×10^5 washed cells (multiplicity of about 1000 PFU/cell) in a total volume of 4 ml, the cells washed, and then plated in the soft agar medium.

For the analysis of chromosome complements, several single colonies were transferred individually from agar layers to liquid medium in Petri dishes containing coverslides. Colchicine (0.5 μg/ml) was added 4 hr before fixation to accumulate metaphases. The cells were caused to swell in hypotonic saline (phosphate-buffered saline 1:5), fixed in acetic acid-alcohol (1:3 v/v), and rapidly air-dried. Preparations were stained with McNeal's tetrachrome or aceto-orcein (Gurr) and mounted.

The following cytological data were then obtained: (*a*) the number of metaphase chromosomes per cell (100 metaphases were analyzed); (*b*) the distribution of chromosome numbers and its mode; (*c*) the number and types of morphological anomalies, i.e., breaks, recombinations, deletions, and abnormal chromosomes; (*d*) the total idiograms of at least 20 metaphases per cell line, classified according to Hsu and Zenzes.[6]

The ability of treated as well as untreated cells to grow as tumors was tested by injecting cell suspensions (0.1 ml of culture medium containing 1×10^4 to 4×10^6 cells), (*a*) subcutaneously in newborn mice and Syrian hamsters, (*b*) subcutaneously in week-old thymectomized Syrian ham-

sters, or (*c*) into the cheek pouch of either normal adult Syrian and Chinese hamsters or (*d*) cortisone-treated 2- to 3-week-old Syrian hamsters. Cheek pouch inoculation of 16–20-day-old Syrian hamsters that had received 0.1 ml cortisone acetate (25 mg/ml) subcutaneously twice weekly, starting on the day of cell inoculation, gave the greatest incidence of tumors[7] and were used routinely. Cell integrity as estimated by neutral red uptake was over 90%. The cheek pouch nodules obtained were removed 10–20 days after implantation and prepared for histological examination, as well as further studies in tissue culture.

Results.—The plating efficiency of cells treated with 0.3–0.5 μg/ml of each of the three carcinogens was 1–5 per cent compared with a value of 10 per cent for untreated cells of the same passage level. Colonies growing in the presence of carcinogen had one of two types of morphology: either a smooth outline similar to that of colonies given by untreated cells, or else an irregular one, with columns of protruding cells giving the colonies a star-shaped appearance. We are at present studying whether changes in colony morphology can be used to assay carcinogenic activity. Cells derived from star-shaped colonies growing at the above concentration of MCA and BP were then, following several passages in the absence of carcinogens, again plated in a higher, normally toxic concentration (2 μg/ml) of these substances and were found to give rise to colonies with the same plating efficiency as untreated controls. Colonies derived from virus-transformed cells grew vigorously in soft agar medium, but did not appear to release virus when plated on susceptible mouse embryo and green monkey kidney cells. Such transformed cells are at present being tested to determine whether they carry virus-specific transplantation antigens.

The chromosomes of cells derived from agar colonies were examined without treatment, and at various times after one or more treatments with the carcinogens, with the solvents alone, after exposure to the viruses, as well as after inoculation into animals, whenever this resulted in growth *in vivo*. All the carcinogen and virus-treated Chinese hamster cell lines appeared to have chromosomal changes which did not occur in cell lines which had not been so treated. The latter, after more than one year of culture in soft agar, retained their pseudodiploidy and an unchanged stem-line karyotype; this was characterized by monosomy in pairs 4 and 8, trisomy in pair 7, and a new chromosome resembling the Y chromosome with a shorter long arm (in addition to their normal Y). Small variations in the total chromosome number were attributable to the presence of a variable number of small metacentric chromosomes in groups 10 and 11. The occasional appearance of a chromosome with a terminal centromere was observed at the 16th passage in agar, apparently arising from a deletion of the short arm of one of the acrocentric[8] chromosomes. Similar observations have been made previously by others.[9, 10]

In contrast to the relative stability of the chromosome complement of untreated cells, a large proportion of virus or carcinogen-treated cells were found to have a minute chromosome (M) as well as a very small telocentric chromosome (ST), half the size of one of the smaller metacentrics (pair 11) (Fig. 1). These two additional chromosomes appeared with a high frequency in the virus-treated cells, often being present in 90 per cent of the metaphases examined, as early as ten cell generations after treatment. Their incidence varied upon further passage but remained higher than 15 per cent, which was the highest frequency found in untreated cells.

After a single treatment with 0.3–0.5 μg/ml of MCA or BP, the most striking change was also the frequent presence of the new small chromosomes, but the karyotype appeared otherwise unmodified. Following a second treatment with these

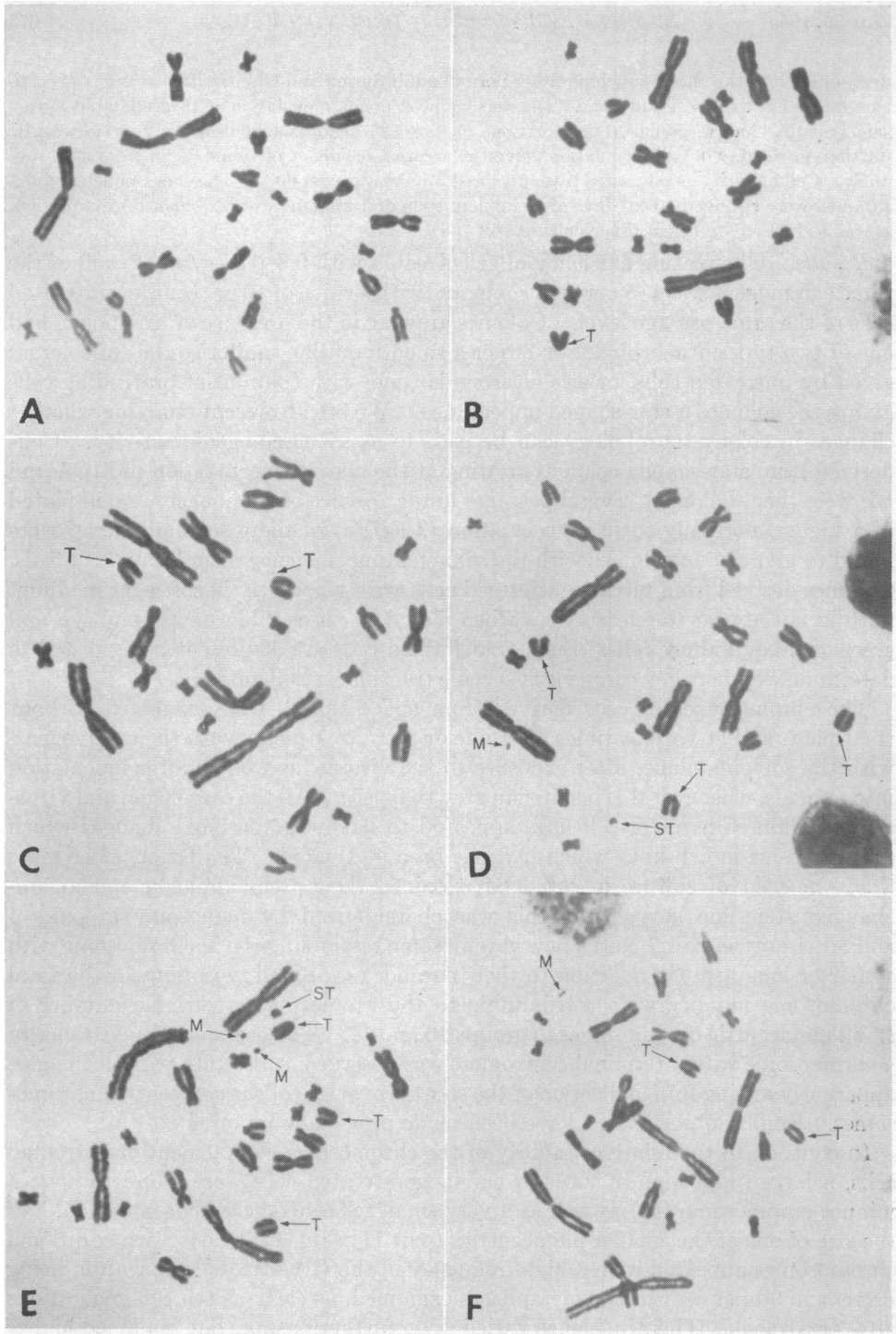

FIG. 1.—Karyotypes of Chinese hamster cells. (*A*) Non-agar-adapted, untreated. (*B*) Agar-adapted, 34 passages in agar; one telocentric chromosome (*T*). (*C*) MCA-treated (0.5 μg/ml and 2 μg/ml) in DMSO solvent; two telocentric chromosomes (*T*). (*D*) BP-treated (0.5 μg/ml and 2 μg/ml) in DMF solvent; one minute (*M*), one small telocentric (*ST*), and three telocentric chromosomes (*T*). (*E*) BP-treated (0.5 μg/ml and 2 μg/ml) in DMSO solvent, *after growth* as cheek pouch tumor and subculture *in vitro;* two minute (*M*) chromosomes, one small telocentric (*ST*) chromosome, and three telocentric (*T*) chromosomes. (*F*) Treated. . . *in vitro;* one minute (*M*) chromosome and two telocentric (*T*) chromosomes.

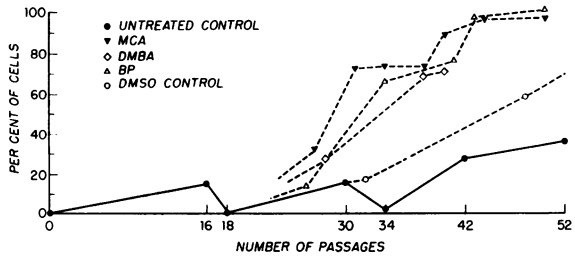

FIG. 2.—Incidence of cells with two or more telocentric (*T*) chromosomes in treated and untreated agar-adapted Chinese hamster cells. Treatments: MCA 0.5 μg/ml at 23rd and 2.0 μg/ml at 29th passage; DMBA 0.5 μg/ml at 24th passage only; BP 0.5 μg/ml at 22nd and 2.0 μg/ml at 33rd passage; DMSO 0.2% at 30th, 31st, and 47th passage.

carcinogens (2 μg/ml) almost all the cells were further modified, now showing two or more telocentric (T) chromosomes (Figs. 1 and 2).

DMBA appeared to be the most toxic of the three hydrocarbons. A single treatment with DMBA (0.5 μg/ml) led to the appearance of M and ST chromosomes as well as two or more T chromosomes per cell, changes which required two treatments with the other carcinogens. In virus-treated cells, the incidence of telocentric (T) chromosomes remained as infrequent as in untreated cells.

Cells treated with DMF had an unchanged chromosome pattern. With DMSO, one treated clone, which was carried for 29 passages and three treatments, showed a chromosomal pattern approaching that seen in the hydrocarbon-treated cells (Fig. 2). It is often difficult to see any specific change in cellular growth pattern following response to carcinogens[11] or to viruses. Since most long-established cell lines grow in a crisscross pattern resembling that of "transformed" cells, morphological alteration by such agents has only been observed with special cell lines retaining contact inhibition[12] or by using primary or secondary cultures made directly from embryonic tissue.[13] We also observed foci of irregularly growing and piling-up cells in secondary cultures of Syrian hamster embryo cells treated *in vitro* with 1 and 2 μg/ml of MCA for 8 days.

When cells were tested for their ability to grow *in vivo* by inoculation into the cheek pouch of Syrian hamsters (Table 1), all the cell lines which had been exposed twice to MCA or BP, or once to DMBA, were able to grow in the alien host. There was a relatively low tumor incidence at the outset. Defendi and Lehman[14] and Todaro *et al.*[15] have both noted that relatively few tumors arise in animals inoculated with cells "transformed" by oncogenic viruses *in vitro*. Only after several alternating passages *in vivo* and *in vitro* could a stable line causing a high frequency of tumors be selected (see *Note added in proof*). In any event, an ability to cause tumors seems to be correlated with the presence of two or more T chromosomes in the majority of the cells inoculated (Fig. 1). Although cells altered by low concentrations of MCA and BP were resistant to higher concentrations of these hydrocarbons (6–8 μg/ml), there was apparently no significant further alteration in either chromosome pattern or the cell's potential to grow *in vivo*. Cheek pouch tumors were also obtained in animals with virus-treated cells (Table 1). The latter did *not*

TABLE 1

CHEEK POUCH TUMORS IN SYRIAN HAMSTERS AFTER INOCULATION OF CHINESE HAMSTER CELLS

	Solvent	Tumors* after inoculation of $1–4 \times 10^6$ cells	Tumors* after inoculation of $1–4 \times 10^5$ cells
Control, no agar	—	0/10	
Control—agar	—	2/20	
Control—agar	DMSO†	1/5	0/3
Control—agar	DMSO‡	2/5	0/5
Control—agar	DMF‡	0/10	
MCA† (0.5 μg)	DMSO	0/5	0/5
DMBA† (0.3 μg)	DMSO	3/19	
MCA‡ (0.5 μg; 2.0 μg)	DMSO	7/20	1/8
BP‡ (0.5 μg; 2.0 μg)	DMSO	4/10	0/8
BP‡ (0.5 μg; 2.0 μg)	DMF	5/10	
SV40	—	4/8	0/3
Polyoma	—	5/17	0/2

DMSO, dimethylsulfoxide; DMF, dimethylformamide.
* All tumors were examined histologically.
† Single exposure.
‡ Two separate exposures.

have the high incidence of T chromosomes noted at the time of inoculation with hydrocarbon-treated cells, yet when fragments from these tumors were explanted into tissue culture, they often showed extra large telocentric chromosomes. However, cells from tumors arising from the injection of virus-treated cells still showed the high incidence of minute and small telocentric chromosomes seen in the original cell lines inoculated. Cells obtained by culturing fragments of tumors derived from carcinogen-treated cells were tested by reinoculation into Syrian hamster cheek pouches. Some cultures resulted in a high incidence of tumors upon reinoculation, others less so.

Most of the cell lines studied, however derived, remained essentially diploid. However, occasional lines obtained either by treating cells *in vitro*, or by subculturing cheek pouch tumors, gave rise to tetraploid cultures. There was a tendency toward tetraploidy in later (~50) passages of all cell lines, untreated as well as treated. Nevertheless, there was no obvious correlation between tetraploidy and ability to grow *in vivo*.

Nodules removed from the cheek pouches of Syrian hamsters receiving inocula of treated cell lines had the microscopic appearance of spindle-cell sarcomas (Fig. 3). They grew as solid masses containing no stroma, infiltrating the soft tissues of the cheek pouch and involving skeletal muscle; tumor cells were frequently seen at the base of the mucosal epithelium, but did not extend into it. Mitotic figures were usually numerous. Little or no inflammatory cell reaction was present at the periphery of the masses. Varying degrees of necrosis were present within the tumors, and in these areas there were sometimes collections of polymorphonuclear leukocytes.

Although there was some variability in the size and shape of the tumor cells, and in the general growth pattern from one tumor to the other, there was no consistent pattern by which tumors derived from the carcinogen or virus-treated material could be distinguished from one another. Likewise, the subsequent transplant generations of these tumors lacked characteristic features.

Discussion.—The ultimate mystery of carcinogenesis lies in how neoplastic cells arise and are maintained. Study of tumors which appear in animals after exposure

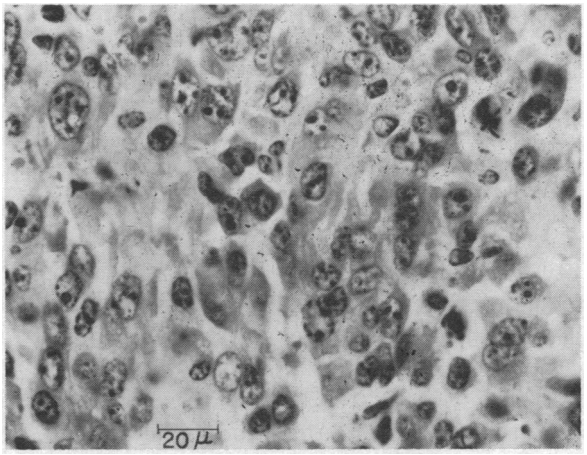

FIG. 3.—Section of tumor grown in hamster cheek pouch. Cells injected were treated twice with MCA *in vitro*. H + E stain.

to tumorigenic viruses or other carcinogens have so far failed to solve this problem. The complexity of the cell populations involved, the length of time before recognizable tumors appear, and the myriad variations encountered have hampered an understanding of the phenomena involved. An attempt has been made in this work to study the events which follow exposure of cells to carcinogenic agents *in vitro*. Berwald and Sachs[13] have already reported the induction of tumors in Syrian hamsters using Syrian hamster embryo cells previously exposed to chemical carcinogens *in vitro*. The cultures involved, however, were heterogeneous with regard to cell content, and their chromosomes too much alike for satisfactory analysis: in the system described here, advantage was taken of the ease with which cloned lines of Chinese hamster cells, showing a conveniently simple karyotype ($2n = 22$), can be selected and propagated. This allowed us to identify chromosome changes associated with virus and carcinogen treatments, and to determine whether any of them may be correlated with the ability of the cells to form tumors when inoculated into an alien host. Since the chromosome complement of cells of the latter (Syrian hamster; $2n = 44$) is clearly different from that of the inoculated cells, it could be determined unequivocally that the malignant growths obtained consist of descendants of the inoculated cells rather than those of the host.

Treatment of cloned Chinese hamster cells with any one of three carcinogenic hydrocarbons, or two oncogenic viruses, gave rise to colonies consisting of cells with an altered chromosome pattern. The most consistent difference between the chromosome complements of chemically treated and untreated cells is the increased incidence of two small additional chromosomes (M and ST) and a twofold increase in the incidence of a telocentric chromosome (T) in the treated cells. Although the present studies are incomplete, they suggest a relationship between the appearance of these chromosome anomalies and the ability of the cells to grow *in vivo:* in all chemically treated lines, when a growth arose in the cheek pouch which was judged malignant by pathological criteria, two telocentric chromosomes were present in a large proportion of the cells prior to inoculation. Thereafter, consistently, two T

chromosomes were also present in tissue cultures made from such growths. Untreated, agar-adapted cells (see Fig. 1*B*) had mostly one T chromosome per cell when inoculated. However, the two tumors obtained from these cells showed two T chromosomes in the majority of the subcultured cells. It must be pointed out that the differences in karyotypes observed between treated and untreated cell lines are entirely quantitative, for there was no chromosomal abnormality that was not also—albeit rarely—observed in untreated cells. The occasional growths produced by "normal" cells may thus be due to the survival and outgrowth in the animal of the rare cells having the necessary genetic constitution.[16] It is also not known at present whether the chromosomal alterations observed *in vitro* are due to direct action by the carcinogenic agent, or whether they result from selective survival in the presence of the agent of cells with the altered karyotype. However, as Figure 2 shows, the considerable interval which elapses between the start of carcinogen treatment and the appearance of chromosome changes in the majority of the population suggests that although the carcinogen may have an effect initially on only a few cells, selection may eventually favor its expression in the majority. The incidence of abnormalities, moreover, seems to increase sharply after a second carcinogen treatment (Fig. 2); after only one treatment, the frequency of abnormalities remains low, even after many cell generations.

That selection does take place is borne out by the observation, both by ourselves and others,[13] that chemically treated cells, besides being tumorigenic, are also able to grow in the presence of hydrocarbon concentrations which would otherwise be lethal. Not only carcinogen-treated, but cells rendered neoplastic by other means, such as virus infection, also resist high hydrocarbon concentrations. Thus, Diamond[3] reported that certain virus-transformed cell lines of rodent origin were more resistant to carcinogenic hydrocarbons than their normal counterparts; these observations were confirmed when we were able to show that SV40-transformed Chinese hamster cells could tolerate a concentration of benzpyrene (4 μg/ml) which was otherwise toxic to normal cells.

Our observations, and those of Defendi and Lehman,[14] of the appearance of minute and small telocentrics in virus (polyoma) transformed cells, suggest that such changes may be the predominant pattern in virus as opposed to carcinogen-induced transformations where two large telocentric chromosomes are frequently observed (Fig. 1*C*). The observation of the Philadelphia (Ph^1) chromosome in cases of chronic granulocytic leukemia in man,[17] as well as the recent description by Moore *et al.*,[18] of both a minute and a small telocentric chromosome in mouse L1210 ascites cells, recalls the similar small chromosomes observed in our treated cell lines.

However, it is as yet impossible to state whether the chromosome anomalies observed are the cause or the result of, or are merely fortuitously associated with, the cells' ability to cause tumors. So we cannot as yet point to any chromosomal "signature" of oncogenic potential.

Note added in proof: After several alternating *in vivo* and *in vitro* passages, carcinogen-treated cells now show a very high tumor incidence when 1×10^4 cells are inoculated into cheek pouches or 2×10^5 cells injected subcutaneously.

We are grateful for the assistance of Mr. M. Pierce, Mrs. A. Berkovitz, and Miss B. P. Carlton. We should also like to thank Dr. D. Hoffman for supplying us with chromatographically purified samples of the hydrocarbons, and Dr. J. Fogh for the strain of SV40 virus.

* This investigation was supported by funds from the National Cancer Institute (grant CA 08748), the Health Research Council of the City of New York (contract U-1096 II A), and grant T-128F from the American Cancer Society and the First National City Bank grant for research from the American Cancer Society.

† These studies were carried out while Dr. Sanders was at Sloan-Kettering Institute for Cancer Research as a recipient of an Alfred P. Sloan Foundation Award in Cancer Research, 1964, on leave from the Virus Research Unit, Medical Research Council Laboratories, Carshalton, Surrey, England (current address).

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