Factors Which Disorganize Microtubules or Microfilaments Increase the Frequency of Cell Transformation by Polyoma Virus

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Griseofulvin, 12-*O*-tetradecanoyl phorbol-13-acetate, melittin, epidermal growth factor, vinblastine, cytochalasin B, podophyllotoxin, colcemid, and colchicine were unable to transform cells but could increase from 8- to 40-fold the frequency of cell transformation by polyoma virus. The 3T3-like cells were resting at confluence and were exposed to the drug only during the 1st week after viral infection. Griseofulvin, a tumor promoter, reduced or increased the frequency of transformation depending on the dose with which the infected cells were treated. The antitumor activity of tumor promoters is discussed.

Polyoma virus and simian virus 40 (SV40) can induce tumors in animals and transform cells in culture. When resting normal cells are infected by polyoma virus or SV40, cellular DNA synthesis is induced, followed by cell division; actin cables are disorganized (8, 34); extensive chromosome alterations, both structural and numerical, are observed (58); and the viral DNA integrates into the cellular DNA. Only a fraction (less than 10%) of the cells displaying those early events become transformed by acquiring a set of new *in vitro* growth properties (namely, the ability to grow in agar) and the capacity to develop into tumors (43, 48). Those cells are referred to as maximal transformants (30).

The mutations which affect the transforming ability of the virus are located within one half of the viral genome. This region is expressed early after infection and in transformed cells. In SV40, it codes for two proteins, the large T (94,000 daltons [94K]) and the small t (17K) (5, 28). In polyoma virus, it codes for three proteins: the large T (100K), the middle t (55K), and the small t (22K) (7, 45). Most maximal transformants contain those viral proteins. However, cells containing both large T and small t but unable to divide in agar or develop into tumors are frequently isolated (31, 33, 41, 42, 44). The continuous presence of viral proteins is therefore not sufficient to render a normal cell a maximal transformant. The establishment of transformation in resting cells requires both large T and small t, but neither seems to be required for the maintenance of transformation (27, 37-40).

Substances which may be involved in the process of chemical carcinogenesis can be clas-

sified into at least two groups: initiators and promoters. The initial studies leading to this distinction were done *in vivo* by applying various substances on the back skin of mice and rabbits and looking for the development of skin tumors. An initiator can induce tumors only when applied at high doses. A promoter by itself cannot induce tumors, but when it is applied after a nontumorigenic dose of an initiator, tumors occur at high frequencies. These results led to the formulation of the two-stage model for carcinogenesis: initiation and promotion (2, 6). Carcinogenesis in two stages also occurs in cell cultures (3, 21).

Since most initiators, but not promoters, are mutagens (20), initiation is postulated to occur by mutation. A recent proposal considers promotion to be the expression of a recessive mutation by abnormal genetic segregation during cell division (14). The "initiated" cell would retain growth control because the mutation is recessive. Promoters would cause the disappearance of the nonmutated allele, and therefore loss of growth control, by inducing abnormalities in chromosome distribution or exchange during mitosis (14).

We proposed that maximal transformation of resting cells by polyoma virus or SV40 is by permanent alteration of cellular growth control which requires only transiently the activities of T and t. It would be accomplished in two stages: initiation (by mutation) followed by promotion. The integration of the viral DNA into the cellular genome, or a misrepaired break in an attempt to integrate, achieves the initiation step. Large T plays a role in this integration, and small t serves as a promoter (27, 37-40). Middle t, which is located in the cell membrane and consists of an enlarged small t (36), enhances

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maximal transformation and acts as a tumor progression factor (36).

Because small *t* disorganizes actin microfilaments (8, 34) and griseofulvin, a tumor promoter, disorganizes microtubules (56), and both induce permanent chromosome numerical aberrations (17, 34), we speculated that tumor promoters act by inducing a partial disorganization of the mitotic spindle, causing chromosome aberrations (40) (microtubules and microfilaments are part of the mitotic spindle).

Agents which affect the mitotic spindle and/or the cytoskeleton as well as known or suspected tumor promoters were selected. The effect of those drugs on the establishment of cell transformation induced by polyoma virus was studied.

MATERIALS AND METHODS

Cells, virus, drugs, and medium. Rat 3T3 cells were previously described (38). Wild-type polyoma virus strain A2 was used. The virus was grown in mouse primary kidney cells (Bethesda Research Laboratories) and purified by isolation from a CsCl gradient. The virus stock suspension was at 5×10^7 PFU/ml in phosphate buffer. 12-*O*-tetradecanoyl phorbol-13-acetate (TPA; mol wt 616.84), 7,12-dimethylbenz(a)anthracene (DMBA; mol wt 256.35), *N*-methyl-*N*-nitroso-*N'*-nitroguanidine (MNNG; mol wt 147.09), and benzo(a)pyrene-*trans*-7,8-dihydrodiol-9,10-epoxy (anti) (BP; mol wt 302.1) were obtained from the National Cancer Institute Chemical Repository, Chicago. Melittin (mol wt 2,848), podophyllotoxin (mol wt 441.41), and vinblastine (mol wt 813.80) were from Sigma Chemical Co. Griseofulvin (mol wt 352.77) was from Aldrich Chemical Co. Colchicine (mol wt 399.5), colcemid (mol wt 371.4), and cytochalasin B (mol wt 479.62) were from Calbiochem. Epidermal growth factor (EGF; mol wt 6,045) was purchased from Collaborative Research, Inc. Stock solutions were in dimethylformamide for all drugs except for EGF and melittin, for which stock solutions were in phosphate buffer.

The medium used for cell culture was Dulbecco modified Eagle medium supplemented with 10% fetal calf serum (GIBCO Laboratories) and antibiotics. The same lot of serum was used in all the experiments.

Transformation assay: effect of drugs. Resting cells at confluence in 6-cm² petri dishes (2.5×10^6 cells/dish) were infected with polyoma virus at a multiplicity of 50 PFU/cell. The infection period was 2 h at 37°C in a total volume of 0.2 ml. Five milliliters of medium containing the drug was then added to every dish. After 7 days, the cells were washed, seeded in 25-cm² flasks, and fed with medium containing no drug. They reached confluence within 3 days. The culture was regularly fed with unsupplemented medium until the experiment was ended, 32 days after viral infection at 33°C.

RESULTS

Effect of tumor promoters, antimetabolic and antimotility agents on cell transfor-

mation by polyoma virus. Resting cells were infected with virus and fed for only 7 days after infection with medium containing various amounts of griseofulvin, EGF, TPA, melittin, vinblastine, podophyllotoxin, colcemid, colchicine, or cytochalasin B. At the end of the exposure period, the cells were washed and regularly fed with medium containing no drug. Dense foci of transformed cells were counted 25 days later. Control cultures were either treated with virus alone or mock infected and exposed to the drug.

The experiment was designed as indicated for the following reasons: (i) with polyoma virus or SV40, only transformed cells derived from resting normal cells appear to be independent of any viral protein for the maintenance of transformation, whereas actively growing cells give rise to transformants which depend on large T for the maintenance of transformation (27, 39); (ii) cell division is induced by the virus in at least 30% of the cells within the 1st week after infection, but a majority reenter the resting state after one or few divisions (34); and (iii) promotion is postulated to require cell division (14). The drug was added so that it could interact with cells induced to divide under restrictive conditions for growth. The treatment with the drug was limited to the 1st week after infection, so only its effect on the establishment of transformation was tested.

The drugs indicated above were chosen because of their properties shown in Table 1. Griseofulvin is a tumor promoter (1); it disorganizes microtubules (56) and the mitotic spindle (19) and induces numerical chromosome aberrations (17). EGF disorganizes microfilaments (8) and is a tumor enhancer (32) (when applied with an initiator, it increases the frequency of tumor induction). TPA is a promoter (3); it disorganizes microfilaments (29) and induces abnormal chromatid exchange (14). Melittin has several cell effects similar to those of TPA (22); it is a suspected, but not yet confirmed, tumor promoter (22). Vinblastine, podophyllotoxin, colcemid, and colchicine disrupt microtubules and the mitotic spindle (19, 24) and induce aberrations in chromosome number or exchange (12, 35, 46). Finally, cytochalasin B disrupts microfilaments (57) and causes polyploidy (4). None of the latter drugs has been reported to be a tumor promoter.

The numbers of dense foci counted in the different cultures are indicated in Tables 2 and 3. Mock-infected cultures did not give rise to a single focus, but formed a homogeneous cell monolayer. The cells infected with virus generated dense foci which could be detected 25 days after viral infection. None of the drugs at any of the indicated concentrations had any effect on

TABLE 1. *Properties of some cytoskeleton-disrupting agents and tumor promoters*

Drug	Medical use	Identified as a promoter or enhancer in the mouse skin test	In vitro properties similar to those of promoters	Components of the cytoskeleton or mitotic spindle affected	Induction of aberrations in chromosome segregation or exchange
Griseofulvin	Antifungal antibiotic	+	+	Microtubules	+
Melittin					
EGF		+		Actin microfilaments	
TPA		+		Actin microfilaments	+
Vinblastine	Antitumor agent			Microtubules	+
Cytochalasin B	Antitumor agent			Actin microfilaments	+
Podophyllotoxin	Antitumor agent			Microtubules	+
Colcemid				Microtubules	+
Colchicine	Antitumor agent			Microtubules	+

TABLE 2. *Effect of tumor promoters, melittin and EGF on cell transformation by polyoma virus*

Transforming agent	No. of dense foci/flask at final drug concn (ng/ml of medium)													
	0	0.24	0.48	0.97	1.95	3.9	7.8	15.6	31.25	62.5	125	250	500	
Mock	n ^{a,b}													
Virus	13 ^c													
Virus + griseo-fulvin				21	>500	262	198	20	15	58	75	114	12	
Griseofulvin				n	n	n	n	n	n	n	n	n	n	
Virus + Melittin			12	17	22	20	30	50	14	16	445	>500		
Melittin			n	n	n	n	n	n	n	n	n	n		
Virus + EGF		30	332	192	70	14	17	13	14	10	12			
EGF		n	n	n	n	n	n	n	n	n	n			
Virus + TPA				18	14	12	10	17	13	14	96	117	214	
TPA				n	n	n	n	n	n	n	n	n	n	

^a n, No dense foci detected.^b Eleven cultures were tested; presence of organic solvent had no effect.^c Average for 11 cultures. The number varied between 11 and 19 foci/flask. Dense foci were counted after the cultures were fixed and stained.TABLE 3. *Effect of antimetabolic and antimotility agents*

Transforming agent	No. of dense foci/flask at final drug concn (pg/ml of medium)										
	0	3.9	7.8	15.6	31.25	62.5	125	250	500	1,000	2,000
Mock	n ^{a,b}										
Virus	13 ^c										
Virus + vinblastine		12	16	10	76	17	20	>500	432	19	12
Vinblastine		n	n	n	n	n	n	n	n	n	n
Virus + cytochalasin B		13	24	16	356	13	28	32	>500	114	14
Cytochalasin B		n	n	n	n	n	n	n	n	n	n
Virus + podophyllotoxin		248	22	26	34	14	13	74	24	24	28
Podophyllotoxin		n	n	n	n	n	n	n	n	n	n
Virus + colcemid		13	50	282	28	12	10	12	17	14	14
Colcemid		n	n	n	n	n	n	n	n	n	n
Virus + colchicine		12	105	13	14	16	54	63	72	16	10
Colchicine		n	n	n	n	n	n	n	n	n	n

^{a-c} See Table 2.

cell viability as determined by the cell cloning efficiency on plastic after treatment. Mock-infected cells treated with the drug alone did not generate any foci. In the cultures infected with virus and treated with certain amounts of drug, an increase in the number of dense foci was

observed compared with the cultures infected with virus alone. All the drugs used were effective. The most effective concentrations were 1.95 ng/ml for griseofulvin, 250 ng/ml for melittin, 0.48 ng/ml for EGF, 500 ng/ml for TPA, 250 pg/ml for vinblastine, 500 pg/ml for cytochalasin B,

3.9 pg/ml for podophyllotoxin, 15.6 pg/ml for colcemid, and 7.8 pg/ml for colchicine. Other concentrations either were less effective or had no apparent effect. For griseofulvin, melittin, vinblastine, cytochalasin B, podophyllotoxin, and colchicine, two groups of effective concentrations separated by apparently ineffective concentrations were observed. In the cultures treated with virus and an effective amount of drug, a fraction of the dense foci induced were detected as early as 18 days after viral infection.

Opposite effects of griseofulvin. Resting cells were infected with virus or mock infected and exposed to griseofulvin for 7 days after infection. The amounts of griseofulvin varied from 50 ng to 50 µg per ml of medium. In the cultures exposed to the highest dose (50 µg/ml), all of the cells were detached from the substrate 3 days after the beginning of the exposure period. Essentially all of the cells exposed to the smaller doses (25 to 0.05 µg/ml) remained attached to the substrate at the end of the exposure period and for the following month.

The number of dense foci found in the different cultures 32 days later is indicated in Table 4. Virus-infected cells generated dense foci, but mock-infected cells did not. In the cultures exposed to the lowest dose of griseofulvin (0.05 µg/ml), a threefold increase in the number of dense foci was observed. A 10-fold-higher dose of griseofulvin (0.5 µg/ml) had no apparent effect on the frequency of cell transformation. Much higher doses of griseofulvin (5, 12.5, and 25 µg/ml) gradually reduced from 3-fold up to more than 30-fold the frequency of cell transformation. Mock-infected cells treated with griseofulvin did not generate any dense focus.

When resting cells were exposed for 7 days to 25 µg of griseofulvin per ml, a concentration which totally inhibited cell transformation by polyoma virus, only a twofold decrease in the cell ability to divide was observed (Table 5).

Griseofulvin had a dual effect on cell transformation *in vitro*; at high doses it inhibited trans-

formation with minimal damage to the normal cells, and at low doses it increased the frequency of cell transformation.

Effect of mutagens. Resting cells were infected with virus and treated with MNNG, BP, or DMBA, all known mutagens (20). A twofold increase in the number of dense foci was observed in the cultures treated with certain amounts of MNNG or BP as compared with the cultures treated with the virus alone (Table 6). On the other hand, no effect of DMBA could be detected. Cells treated with the mutagen alone did not generate any dense foci. The most effective concentration was 10^{-5} M for both MNNG and BP. At this concentration, greater than 99% of the treated cells were attached to the substrate at the end of the exposure period, but a 50% reduction in the cell ability to divide was observed (Table 5). The twofold increase in the frequency of cell transformation could therefore be considered as a fourfold increase, since half of the treated cells were alive but unable to divide. With DMBA there was no detectable effect, possibly reflecting the inability of 3T3 rat cells to modify DMBA into an active form. Various mutagens were previously reported to en-

TABLE 5. Ability of cells to divide after treatment with different drugs^a: cloning efficiency on plastic

Seed- ing ^b density	Un- treated cells	Total no. of clones/flask			
		Cells treated with:			
		Griseofulvin		MNNG, 10 ⁻⁵ M	BP, 10 ⁻⁵ M
		12.5 µg/ ml	25 µg/ ml		
1,000	55	32	23	28	22
500	21	19	13	16	19
250	13	12	5	6	5

^a Resting cells were exposed to the drug for 7 days.

^b Number of cells plated per 25-cm² flask.

TABLE 6. Effect of mutagens

Transforming agent	No. of dense foci/flask at final carcinogen concn (M) of:				
	0	10 ⁻⁸	10 ⁻⁷	10 ⁻⁶	10 ⁻⁵
Mock	n ^a				
Virus	51				
Virus + MNNG		42	37	47	105
MNNG		n	n	n	n
Virus + BP		35	43	74	111
BP		n	n	n	n
Virus + DMBA		40	35	— ^b	—
DMBA		n	n	—	—

^a No dense foci detected.

^b Total cell detachment was observed within 3 days of treatment with the mutagen.

TABLE 4. Dual effect of griseofulvin

Transforming agent	No. of dense foci/flask at final drug concn (µg/ml of medium)						
	0	0.05	0.5	5	12.5	25	50
Mock	n ^a						
Virus	34						
Virus + griseofulvin		112	41	11	6	0	— ^b
Griseofulvin		n	n	n	n	n	—

^a n, No dense foci detected.

^b —, Total cell detachment within 3 days of exposure to the drug.

hance the frequency of cell transformation induced by polyoma virus or SV40 (10, 26, 47, 52).

DISCUSSION

Nonmutagens such as griseofulvin, TPA, melittin, EGF, vinblastine, podophyllotoxin, cytochalasin B, colcemid, and colchicine increased the frequency of cell transformation induced by polyoma virus. A short period of exposure to those agents, early after viral infection, was sufficient to reveal this activity. The drugs by themselves were unable to induce cell transformation. Known mutagens had a similar but smaller effect. The 3T3-like rat cells were resting at the time of infection and were under restrictive conditions for normal cell growth for the following 7 days, when the drug was present in the medium.

The effect on the frequency of transformation varied among the different drugs (Table 7). Griseofulvin, melittin, vinblastine, and cytochalasin B were the most effective, judging by the net increase without taking into account the doses to which the cells were exposed.

Agents selected for their property of interacting with microtubules, microfilaments, or the mitotic spindle (vinblastine, podophyllotoxin, cytochalasin B, colcemid, and colchicine) all acted like the known tumor promoters (TPA and griseofulvin). They increased from 8- to at least 40-fold the frequency of virus-induced transformation. Furthermore, those antimitotic-antimotility agents induce chromosome aberrations during mitosis (4, 12, 35, 46). TPA and griseofulvin also interact with microfilaments or

microtubules and induce chromosome aberrations (14, 17, 29, 56). Taken together, these data suggest that: (i) the enhancing activity detected in this assay may involve the mitotic spindle and its role in chromosome segregation; and (ii) the process of tumor promotion may have several aspects in common with the process underlying this enhancing activity. Other possibilities are considered below. The formal demonstration of a tumor promotion activity for the above-cited drugs not reported as tumor promoters requires that a gap in time exist between treatment with virus and treatment with the drug. Those experiments are presently underway. It is of interest to note that the concentration of TPA which was effective in the assay described (0.1–0.5 $\mu\text{g}/\text{ml}$) is within the range of doses required to demonstrate the tumor promotion activity of TPA in vitro (3, 21) or the activity which induces sister chromatid exchange (14). TPA appeared to be slightly less effective, at the highest concentration used (0.5 $\mu\text{g}/\text{ml}$), than melittin or EGF. TPA is usually the most potent tumor promoter. We do not consider this difference highly significant. A more effective dose of TPA may be found at higher concentrations, and the different cells and assays may differ in sensitivity.

Alternatively, all of these drugs may share another unknown property which is actually responsible for the activity detected in this assay, a possibility that cannot be ruled out at the present time. It is difficult to argue that this other property is the induction of cell division in resting cells, since at the multiplicity of infection used the virus alone is able to induce several rounds of cell division in at least 30% of the infected cells (34). Another possibility is that these agents increase the efficiency of integration of the viral DNA into the cellular genome and therefore raise the frequency of transformation. This explanation was proposed to account for the enhancement of viral transformation by mutagens (10). At the present time, it remains a formal possibility for nonmutagens. A total of 26 foci of transformed cells induced by treatment with both polyoma virus and griseofulvin were isolated. The presence of viral proteins in those transformants as well as their growth properties are presently being studied. Still another possibility is that these agents activate one or several cryptic oncogenes only when combined temporarily with some function of polyoma virus.

Another group of investigators (18) could not reproduce the results reported (14, 23) concerning the induction of sister chromatid exchange by TPA. The reasons for this discrepancy remain to be determined. On the other hand, when

TABLE 7. Effect of various drugs on the frequency of polyoma virus-induced cell transformation

Drug	Most effective concn ^a	Fold increase of virus-induced cell transformation ^b
Griseofulvin	1.95 ng/ml (5.54)	>38
Melittin	250 ng/ml (87)	>38
EGF	0.48 ng/ml (0.08)	25.5
TPA	500 ng/ml (811)	16.4
Vinblastine	250 pg/ml (0.31)	>38
Cytochalasin B	500 pg/ml (1)	>38
Podophyllotoxin	3.9 pg/ml (0.01)	19
Colcemid	15.6 pg/ml (0.04)	21.7
Colchicine	7.8 pg/ml (0.02)	8
MNNG	1.5 $\mu\text{g}/\text{ml}$ (10,000)	2–4
BP	3 $\mu\text{g}/\text{ml}$ (10,000)	2–4

^a 2.5×10^5 cells were exposed for 7 days after infection to 5 ml of medium containing the drug. Nanomolar concentration is given in parentheses.

^b Number of transformants obtained after treatment with virus plus drug divided by number of transformants obtained after treatment with virus alone.

TPA was added to cells irradiated by UV, an increase in the frequency of induced mutants was observed (53). This result is consistent with the hypothesis that TPA causes expression of recessive mutations (14).

Griseofulvin at high doses inhibited cell transformation by polyoma virus. At low doses, it increased the frequency of cell transformation. Such a dual effect has been reported in vivo. Griseofulvin given to animals orally had a promoting activity on initiated skin tumors (1). When applied directly on the skin, it had no promoting activity and could actually inhibit carcinogenesis promoted by TPA (54). Several other tumor promoters or enhancers (mezelein, croton oil factor B3, phenobarbital) also have an antitumor activity (11, 15, 16, 25). Vinblastine, podophyllotoxin, colchicine, and cytochalasin B are used in vivo as antitumor agents because of their antimitotic activity or capacity to alter cell motility (9, 13). Colcemid and colchicine, used in vitro, block mitosis at concentrations of at least 20 ng/ml. These antitumor-antimitotic-antimotility drugs behave like the tumor promoters (TPA and griseofulvin) in the assay reported here at doses 100- to 1,000-fold less than those required for antimitotic activity. The double effect of tumor promoters may be related to their interaction with components of the mitotic spindle. At high doses they would ruin the mitotic spindle, preventing mitosis; at low doses they would interfere only slightly with the organization of the spindle, causing chromosome aberrations which may result in promotion.

The disrupting effect on the cytoskeleton of agents such as vinblastine, cytochalasin B, or colchicine can be detected by indirect immunofluorescence. Concentrations of at least 0.3 $\mu\text{g}/\text{ml}$ up to 10 $\mu\text{g}/\text{ml}$ are required to detect the disorganization of microtubules or microfilaments. The concentrations of those agents which were effective in our assay are 1,000- to 100,000-fold lower. It is almost certain that at those concentrations the above-cited agents have no detectable effect by immunofluorescence on the general organization of the cytoskeleton.

The results reported are consistent but certainly do not establish that small t acts as a promoter and that large T plays a role in initiation. Two sets of results obtained by other investigators encourage further studies in this direction. Theile et al. (49) had reported that SV40 induces a variety of somatic mutations in cell cultures. More recently it was shown that this mutagenic activity increases with the multiplicity of infection, is higher when cells are treated with a mutagen and virus, and is dependent on the activity of large T (temperature-sensitive mutants of large T do not induce mutations at

high temperature). Furthermore, greater than 75% of the SV40-induced mutants were found to contain viral proteins (and probably integrated viral DNA) (50, 51). Cells infected by wild-type SV40 generate minimal transformants and maximal transformants (30, 31, 33, 44, 55). Minimal transformants differ from normal cells by the ability to grow in medium containing low amounts of serum factors. However, they grow to a low saturation, do not grow in agar, and do not form tumors (30, 31, 41, 42). They cannot be detected by the dense focus assay. SV40 cannot induce dense foci in resting cells without small t (40). However, without small t, minimal transformants were generated from cells subjected to restrictive conditions for normal cell growth early after infection (42). It is tempting to speculate that at least some minimal transformants are initiated cells with a partial loss of growth control which can be converted to maximal transformants by small t or another promoter.

Finally, the assay described may prove to be useful for a rapid detection of tumor promoters.

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