Taxol-dependent Mutants of Chinese Hamster Ovary Cells with Alterations in α- and β-Tubulin

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Abstract. Chinese hamster ovary cell mutants resistant to the microtubule stabilizing drug taxol were isolated in a single step. Of these 139 drug-resistant mutants, 59 exhibit an absolute requirement for taxol for normal growth and division, 13 have a partial requirement, and 69 grow normally without the drug. Two-dimensional gel analysis of whole cell proteins reveal "extra" spots representing altered tubulins in 13 of the mutants. Six of these have an altered α-tubulin and seven have an altered β-tubulin. Cells with an absolute dependence on taxol become large and multinucleated when deprived of the drug. In contrast, partially dependent cells exhibit some multinucleation, but most cells appear normal. In one mutant that has an absolute dependence on taxol, the cells appear to die more quickly and their nuclei do not increase in size or number.

As previously found for another taxol-dependent mutant (Cabral, F., 1983, *J. Cell. Biol.,* 97:22–29), the taxol dependence of the mutants described in this paper behaves recessively in somatic cell hybrids, and the cells are more susceptible to being killed by colcemid than are the wild-type parental cells. When compared with wild-type cells, taxol-dependent mutants have normal arrays of cytoplasmic microtubules but form much smaller mitotic spindles in the presence of taxol. When deprived of the drug, however, these mutants cannot complete assembly of the mitotic spindle apparatus, as judged by tubulin immunofluorescence. Thus, the defects leading to taxol dependence in these mutants with defined alterations in α- and β-tubulin appear to result from the cell's inability to form a functional mitotic spindle. Reversion analysis indicates that the properties of at least one α-tubulin mutant are conferred by the altered tubulin seen on two-dimensional gels.

MICROTUBULES are involved in many important cellular functions including mitosis, particle and organelle transport by saltatory motion, and secretion (11, 25). Much has been learned about microtubule assembly and function through the use of morphological techniques and specific microtubule-inhibiting drugs. Our understanding of microtubule assembly and organization has also been furthered by in vitro polymerization studies and by the ability to visualize arrays of these structures in whole cells with fluorescently labeled antibodies. Another approach that has been enormously successful in studying biological processes is the combination of genetics and biochemistry. This type of approach has only recently been used to study the cytoskeleton and should provide much useful information about the control of microtubule assembly and function, especially when combined with the morphological techniques available.

Early attempts were made to select yeast and *Chlamydomonas* cells resistant to microtubule-inhibiting drugs (1, 15, 29). The specific defects responsible for the drug resistance phenotype, however, could not be demonstrated. Later investigations with the fungus *Aspergillus* proved more useful. Mutants resistant to benomyl were found to have alterations in β-tubulin, and a revertant of one of these strains was found to have an altered α-tubulin (18, 26). Using these mutants, the authors were able to demonstrate that nuclear movement in that organism is microtubule dependent (19, 20). In mammalian cells, Ling and Thompson first isolated colchicine-resistant mutants in Chinese hamster ovary (CHO) cells[1] (16). Although these mutants had defects in membrane permeability, later mutants isolated by the same group were shown to have altered colcemid binding affinity, and one had an alteration in α-tubulin (14, 17). In our laboratory we have employed a similar approach, i.e., selecting cells resistant to microtubule-active drugs to obtain CHO mutants with distinct alterations in α- and β-tubulin (4, 6, 7, 9, 21, 22).

Most microtubule inhibitors cause microtubules to break down, but one, taxol, stabilizes microtubules and promotes their assembly (23, 24). Among CHO cells resistant to taxol, a mutant was discovered that required the continuous presence of the drug for cell division and long term viability (3, 10). This mutant has no altered forms of α- or β-tubulin detectable by two-dimensional gel analysis. The biochemical lesion responsible for the phenotype of this mutant is still unknown.

We now report the isolation of many new taxol-requiring CHO cell lines, some of which carry altered forms of α- and

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[1] *Abbreviations used in this paper:* CHO, Chinese hamster ovary; HAT, hypoxanthine-aminopterin-thymidine.

β-tubulin. We also present genetic and morphological characterizations of some of these strains.

Materials and Methods

Sources of Drugs and Antibodies

Taxol (NSC) (125973-N1 lot FB1021-58-61) was kindly supplied by the Developmental Therapeutics Branch of the National Cancer Institute. Colcemid was obtained from Gibco (Grand Island, NY). Puromycin, ouabain, and aminopterin were obtained from Sigma Chemical Co. (St. Louis, MO). Affinity-purified anti-tubulin antibodies were the kind gift of Dr. Bill R. Brinkley (University of Alabama, Birmingham, AL).

Mutant Isolation

The CHO cell line used in these studies and the conditions for its growth have been previously described (27). To isolate mutants, cells were plated onto 100-mm tissue culture dishes (5 × 10⁶ cells/dish) containing 10 ml minimum essential medium (alpha-modification, Flow Laboratories Inc., McLean, VA) supplemented with 10% fetal bovine serum (MA Bioproducts, Walkersville, MD), 2 mM glutamine, 50 U/ml of penicillin, and 50 μg/ml streptomycin (Flow Laboratories Inc.). The dishes were then incubated at 37°C for 1 h to allow cells to attach. The cells were rinsed twice with Dulbecco's phosphate-buffered saline (PBS) and covered with 5 ml more of PBS. They were then irradiated for 13 s at a distance of 24 in with a 15-W General Electric germicidal lamp. The mutagenized cells were allowed to recover and grow in flasks for 3 d before being plated onto selective medium. The surviving fraction of mutagenized cells was ~20%.

Taxol-resistant cells were selected by plating 5 × 10⁵ mutagenized cells onto each of 14 100-mm dishes with 20 ml complete medium and 0.3 μg/ml taxol (diluted from a stock solution containing 1 mg/ml in dimethyl sulfoxide). After 7 d at 37°C, 139 of the surviving clones were isolated and grown in medium containing 0.2 μg/ml taxol. In all further procedures, cells grown in the presence of taxol were grown in medium containing 0.2 μg/ml of the drug except where indicated otherwise. The clones were then subjected to three different screening procedures. Cells were tested for their ability to grow in complete medium without taxol to determine if they were taxol dependent. They were also grown in medium containing 7 μg/ml of puromycin to look for cross-resistance to this drug, thereby allowing us to detect putative permeability mutants (2, 9, 22). Finally, whole cell lysates of each clone were analyzed by two-dimensional gel electrophoresis to identify those that contain tubulin with an altered electrophoretic mobility.

Other Procedures

Two-dimensonal gel analysis (5), growth measurements, viability measurements and phase microscopy (3), and antitubulin immunofluorescence (10) were performed as described earlier. Somatic cell hybridizations were performed by fusing mutant cells with a wild-type strain (10512) which contains markers for 8-azaguanine (hypoxanthine-aminopterin-thymidine [HAT] sensitivity) and ouabain resistance as described earlier (13). Approximately 10⁶ cells of each mutant strain (and also the wild-type parent) were plated onto a 60-mm dish with an equal number of cells from strain 10512 and allowed to attach. Cells were fused with polyethylene glycol 1000, incubated overnight in medium, trypsinized, resuspended in 22 ml medium, and aliquoted into dishes (0.5 ml into each of four dishes, 5 ml into each of another four dishes) containing various amounts of taxol. The cells were again incubated overnight, and the medium was then replaced with HAT/ouabain medium containing the same amount of taxol. The cells were incubated for 7 d and stained with methylene blue, and colonies were counted. Before cells were stained, a few colonies were picked and grown in HAT/ouabain medium with taxol for karyotyping.

For selection of revertants, cultures of mutant cells growing in taxol-containing medium were trypsinized, plated onto 100-mm dishes at a density of 5 × 10⁵ cells in medium without taxol, and incubated for 1 wk. Several growing colonies were picked and grown in fresh medium, then subcloned.

Results

Taxol-dependent Clones Are Found at High Frequency Among Cells Selected for Resistance to Taxol; Permeability Mutants Are Rare

Taxol-resistant clones were found at a frequency of 2.5 × 10⁻⁴ among cells mutagenized with ultraviolet light. The results of

our selection are summarized in Fig. 1. From 13 different dishes, 139 taxol-resistant clones were isolated and grown in taxol containing medium. They were then screened for taxol dependence by plating in taxol-free medium. Of the 139 clones, 57 exhibited an absolute requirement for taxol; i.e., all cells ceased dividing and became large and multinucleated within 2 d of growth without taxol. Also, 13 additional clones had a partial requirement for the drug. These cells could grow and form colonies in the absence of taxol, albeit much more
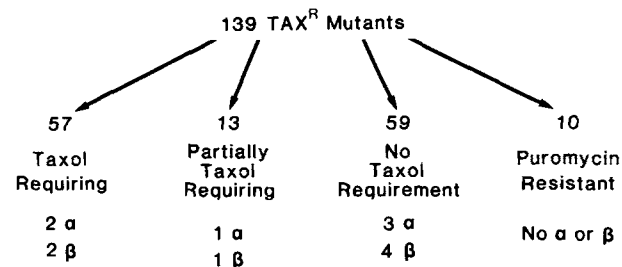

Figure 1. Results of selection of ultraviolet-irradiated CHO cells for resistance to 0.3 μg/ml of taxol. Mutants were tested for drug resistance and requirement as described in Results.
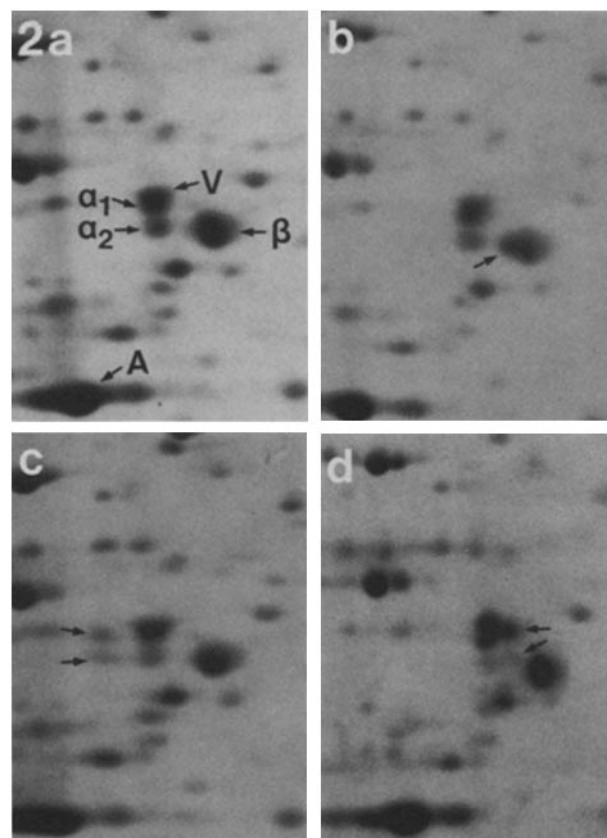

Figure 2. Autoradiograms of the tubulin region of two-dimensional gels of [³⁵S]methionine-labeled whole cell proteins of taxol-requiring CHO cell mutants. Cells were labeled for 30 min in [³⁵S]methionine, lysed in SDS, and analyzed by two-dimensional gel electrophoresis as previously described (9). (*a*) Wild-type pattern; (*b*) β-tubulin mutant Tax 11-3; (*c*) α-tubulin mutant Tax 11-6; (*d*) α-tubulin mutant Tax 2-3. In *a*, α₁ and α₂ are spots representing α-tubulin; β, β-tubulin; V, vimentin; A, actin. In *b-d*, arrows indicate spots representing altered forms of tubulin proteins. Note that α-tubulin resolves as two spots on our gels. These do not represent distinct gene products but probably result from a posttranslational modification.

slowly than those grown in the presence of taxol. Many multinucleated cells could be found among these clones after 2 d of growth in the absence of the drug.

Cells were also screened for resistance to puromycin, a drug that inhibits protein synthesis and has no specific effects on microtubules. This procedure identifies cells with membrane permeability defects that have been found to occur at high frequency among cells selected for resistance to microtubule inhibitors. Such cells are cross-resistant to a wide variety of drugs and are thus unlikely to have specific defects in microtubules (2, 9, 22). Among the 139 taxol-resistant clones, only 10 were resistant to puromycin at a level that kills wild-type cells (7 μg/ml). It is not surprising that none of the puromycin-resistant clones required taxol for normal growth.

Mutants Containing Altered α- and β-Tubulins Are Found among Both Taxol-resistant and -dependent Clones

For initial screening, whole cell lysates of the 139 clones were prepared in hot SDS sample buffer and analyzed by two-dimensional gel electrophoresis (5) followed by silver staining (31). Clones carrying altered forms of α- or β-tubulin were recloned in soft agar, grown, pulsed with [^{35}S]methionine, lysed in SDS buffer, and run again on two-dimensional gels. Among the 139 taxol-resistant clones, six were found to have two "new" spots representing mutant α-tubulin migrating next to the two wild-type α-tubulin spots; seven have a new spot representing mutant β-tubulin next to wild-type β-tubulin. The presence of these "new" spots is consistent with results previously reported from this laboratory (9, 22) for

CHO mutants with altered tubulins.

Of the 13 clones that exhibit tubulin with an altered electrophoretic mobility, seven (three α, four β) were found among the 59 taxol-resistant but non-requiring clones and six (three α, three β) were among the 70 taxol-dependent mutants (Fig. 1). Representative autoradiograms of the tubulin regions from two-dimensional gels of some of these mutants are shown in Fig. 2. Altogether four distinct patterns among the six mutants with an altered α-tubulin and two distinct patterns among the seven mutants with an altered β-tubulin have been identified. As expected, none of the puromycin-resistant clones carried an altered tubulin.

Some Tubulin Mutants Require Taxol for Cell Division

Two taxol-dependent clones, one containing an altered α-tubulin (Tax 11-6) and the other carrying an altered β-tubulin (Tax 11-3), were chosen for further characterization. For some studies, other clones were also used. Growth of these two clones and of wild-type cells was measured in the presence and absence of taxol. Wild-type cells grew rapidly with a doubling time of 12 h in the absence of taxol, but ceased growing and dividing after 24 h in the presence of the drug (Fig. 3a). Tax 11-6, which contains an altered α-tubulin, showed a nearly opposite growth pattern from the wild-type cells. These cells grew with a doubling time of ~14 h in the presence of taxol. When deprived of the drug, however, they stopped growing after 24 h (Fig. 3b). Tax 11-3, a β-tubulin mutant, also grew well with taxol present but was growth inhibited in the absence of the drug (not shown). Another α-

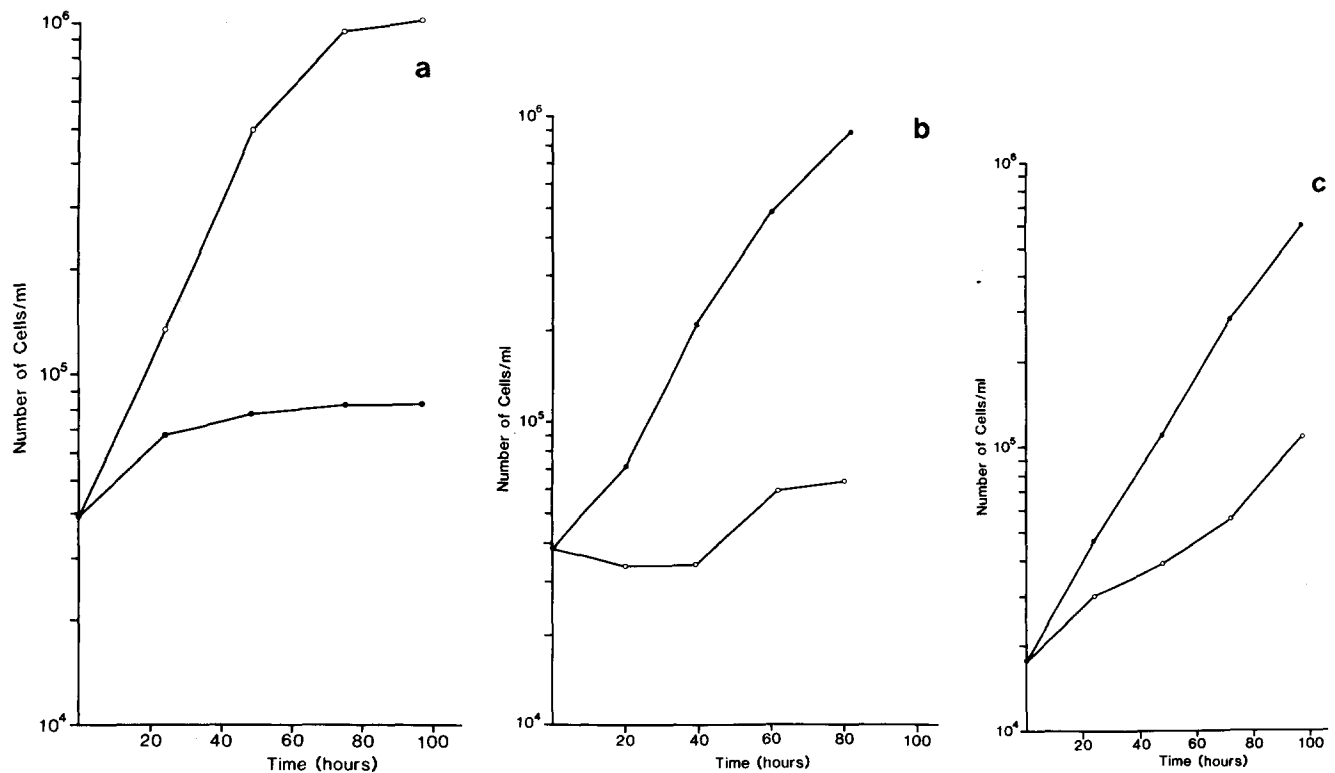*Figure 3.* Growth curves of wild-type cells and taxol-requiring tubulin mutants in the absence of taxol (O) and the presence of 0.3 μg/ml of taxol ($\bullet$). Cells were trypsinized from replicate wells of a 24-well dish at various times and counted as described previously (3). The vertical axis represents the number of cells in a given well after incubation at 37°C for a given period. (a) Wild-type cells; (b) fully dependent mutant Tax 11-6; (c) partially dependent mutant Tax 2-3.

tubulin mutant, Tax 2-3, had a partial requirement for taxol. As shown in Fig. 3c, this mutant grew with a doubling time of ~13 h in the presence of taxol, but in the absence of the drug, the doubling time was increased to ~37 h. Growth of this mutant was never completely inhibited in the absence of the drug, unlike growth of the other mutants.

The effect of taxol deprivation on the growth of the fully taxol-dependent mutants is irreversible and similar to the effect on the original taxol requiring mutant, Tax 18 (3). We measured the loss of cell viability using a method described earlier (3), i.e., by measuring the plating efficiency of the cells after various periods of taxol deprivation. Fig. 4 shows that Tax 11-3 and 11-6, like the previously described mutant Tax 18, lose the ability to form plainly visible macroscopic colonies when taxol is removed from the medium for more than a few hours. Cells of Tax 11-3 are more sensitive to taxol deprivation than are those of Tax 11-6.

Taxol-dependent Mutants Deprived of Taxol Assume Altered Morphologies

In all strains examined, mutants that are completely dependent upon taxol begin to exhibit gross morphological changes after 24 h of growth in the absence of the drug. Fig. 5a is a phase-contrast micrograph of wild-type cells growing without taxol in the medium. Fig. 5b shows the β-tubulin mutant, Tax 2-4, growing in the presence of taxol; these cells appear similar to those shown in Fig. 5a. Of our taxol-dependent mutants, Tax 2-4 is the most sensitive to taxol deprivation. After 24 h without the drug, some cells became multinucleated, but most stopped dividing altogether and began to die (Fig. 5c). This effect became even more pronounced after 48 h without the drug (Fig. 5d). A more typical situation is found with Tax 11-6, which resembles the previously described mutant Tax 18 when deprived of taxol (3). After 24 h without the drug, most of the cells became large and multinucleated (Fig. 5e). Mitotic cells appeared larger and contained more chromosomes than those grown in the presence of the drug. After 48 h without the drug, interphase cells were even larger and contained more micronuclei (Fig. 5f). Mitotic cells were also larger than those deprived of taxol for 24 h and had many chromosomes. We also examined the morphologies of our partially dependent mutants after taxol deprivation. Fig. 5g shows cells of Tax 2-3 after 24 h without taxol. Some cells became multinucleated, but most retained their normal morphology. After 48 h without taxol, the affected cells were even larger and contained many nuclei, but most cells still appeared normal.

Taxol Dependence in Somatic Cell Hybrids Is Recessive

To test whether the taxol-dependent phenotype is dominant or recessive, cell hybrids were constructed between wild-type parental cells and the mutant strains as described in Materials and Methods. As shown in Table I, all of the hybridizations produced large numbers of colonies when plated on HAT/ ouabain medium without taxol. This result would be expected if the taxol dependence phenotype were recessive. A few hybrids of mutant and wild-type cells grew at the intermediate (0.05 and 0.1 μg/ml) concentrations of taxol, but very few of the wild-type/wild-type hybrids grew at these concentrations.
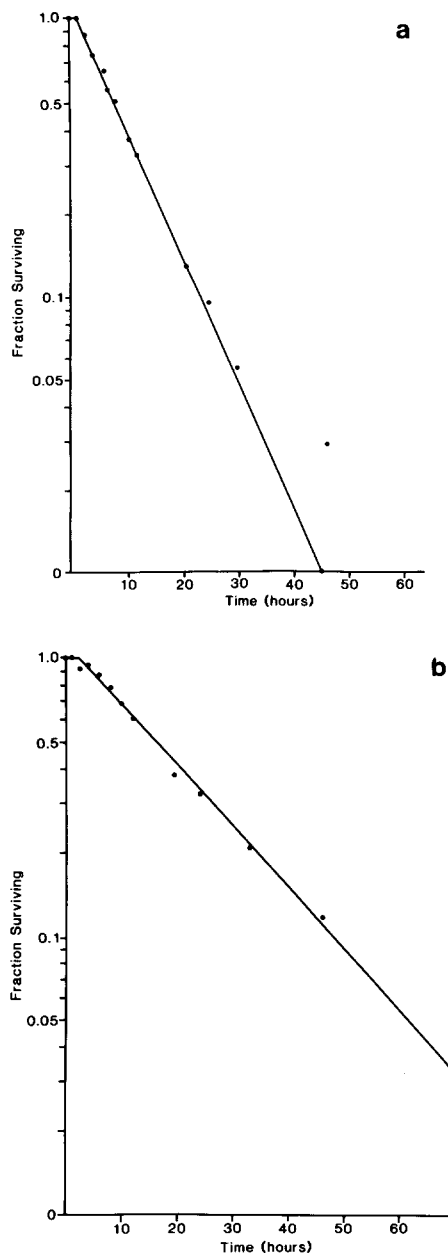

Figure 4. Viability of taxol requiring tubulin mutants Tax 11-3 (a) and Tax 11-6 (b) after the removal of taxol. Approximately 500 mutant cells were plated onto replicate 60-mm dishes and exposed to taxol-free medium for various periods before taxol was added back. Colonies were allowed to form (7–10 d at 37°C) and then counted. The fraction surviving is the number of colonies able to form on a dish deprived of taxol for a given period, divided by the number of colonies able to form on a dish in which cells were kept in taxol-containing medium throughout the experiment.

This result indicates that the phenotype for taxol resistance is weakly co-dominant.

Taxol-dependent Cells Have Increased Resistance to Taxol but Increased Sensitivity to Colcemid

Our taxol-requiring CHO strains were selected for resistance to taxol at a concentration of 0.3 μg/ml. To determine the levels of resistance to, and dependence on, taxol, ~100 cells of each strain to be tested were added to wells of 24-well
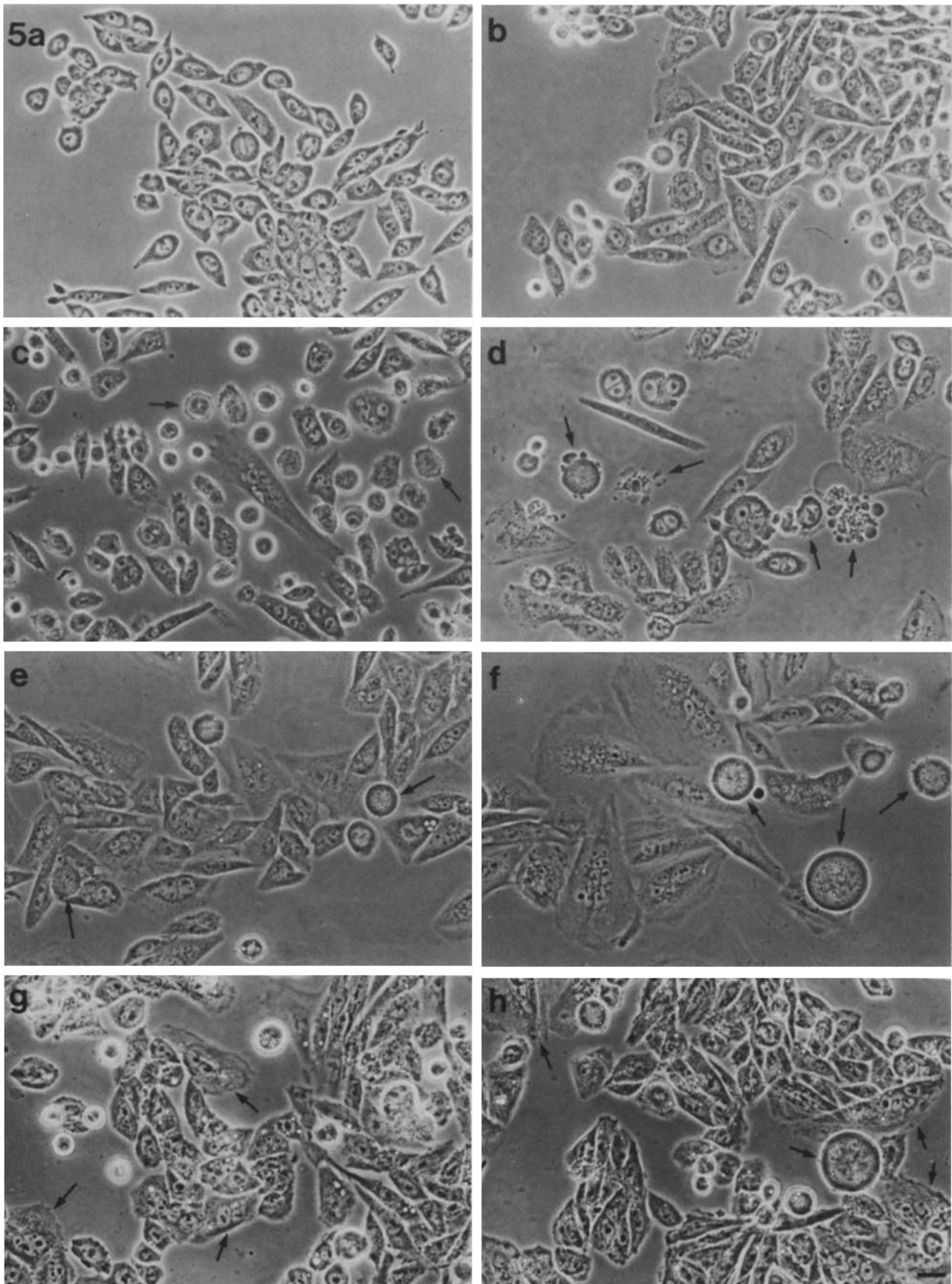
Figure 5. Phase-contrast micrographs of taxol-requiring mutants cultured in the continuous presence of taxol or deprived of taxol for 24 or 48 hours. Cells were photographed on a Nikon Diaphot microscope with a 40× objective lens. (*a*) Wild-type parental cells grown without taxol; (*b–d*) fully requiring mutant Tax 2-4 growing in medium containing 0.2 μg/ml of taxol (*b*) or deprived of taxol for 24 (*c*) or 48 (*d*) h. (*e* and *f*) Fully requiring mutant Tax 11-6 after growth without taxol for 24 (*e*) or 48 (*f*) h. (*g* and *h*) Partially requiring mutant Tax 2-3 after growth without taxol for 24 (*g*) or 48 (*h*) h. Note that almost all cells in *b* have normal morphologies although one or two are multinucleated. In *c*, note the increase in number of multinucleated cells and altered morphologies of some cells (arrows). In *d*, note the necrotic appearance of several cells (arrow). In *e* and *f*, most interphase and mitotic cells (arrows) are larger and are either multinucleated or contain many chromosomes. In *g* and *h*, only some cells suffer the effects of taxol deprivation (arrows), whereas most appear normal. Bar, 2 μm.

Table I. Fusions of Wild-type Cells with Taxol-requiring Mutants

Table I. Fusions of Wild-type Cells with Taxol-requiring Mutants

	Strain fused with wild type							
	WT		Tax 2-4		Tax 11-3		Tax 11-6	
Fraction of fused cells	0.9	0.1	0.9	0.1	0.9	0.1	0.9	0.1
Concentration of taxol (µg/ml)								
0	175	107	510	145	729	142	380	76
0.05	2	0	440	31	80	5	181	4
0.1	0	0	40	6	7	1	3	1
0.2	0	0	0	0	0	0	0	0

5×10^5 cells of wild-type or a taxol-requiring strain were mixed on a culture dish with 5×10^5 cells of a wild-type containing HAT[S] and Ouabain[R] markers. Cells were fused with polyethylene glycol, plated in HAT medium containing ouabain, and given concentrations of taxol. Plates were incubated for 7 d and stained with methylene blue, and colonies were counted.
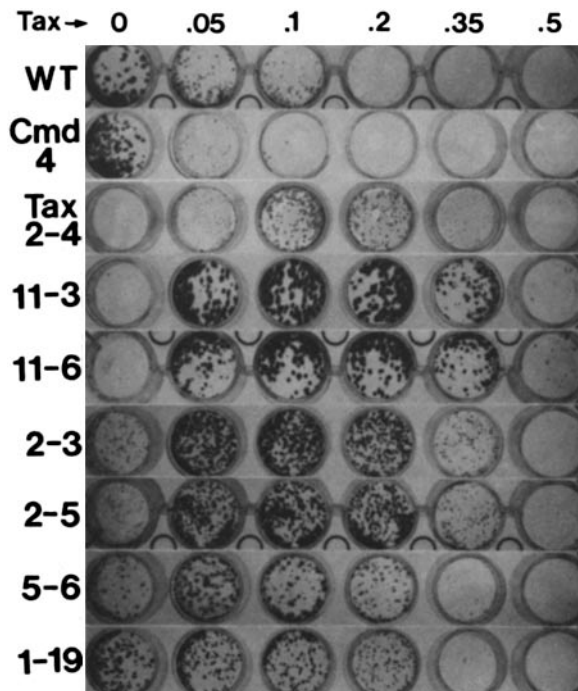

Figure 6. Growth of taxol-resistant and -requiring mutants in taxol. Approximately 100 cells were added to wells containing medium with varying concentrations of taxol, grown for 7–8 d at 37°C, and stained with methylene blue. Taxol concentrations are given as micrograms taxol per milliliter medium. *WT*, wild-type parental cells; Cmd 4, a colcemid resistant β-tubulin mutant; Tax 2-4, a fully taxol-requiring β-tubulin mutant; 11-3, another fully taxol-requiring β-tubulin mutant; 11-6, a fully taxol-requiring α-tubulin mutant; 2-3, a partially taxol-requiring α-tubulin mutant; 5-6, a taxol-resistant (non-requiring) α-tubulin mutant; 1-19, a taxol-resistant (non-requiring) β-tubulin mutant.

dishes containing medium with various concentrations of the drug. As shown in Fig. 6, the taxol-resistant mutants are from two- to fourfold more resistant to the drug than are wild-type cells. Some strains, such as 2-4, 11-3, and 11-6, which we define as having an absolute requirement for taxol, exhibit no growth in the absence of the drug, whereas strains 2-3 and 2-5, which we have designated as partially requiring mutants, exhibit only limited growth and form small colonies without

taxol. One mutant, 2-4, does not grow well even at 0.05 µg/ml taxol and thus appears to have a more severe defect than the other strains. Similar patterns of taxol resistance or dependence are seen for cells with alterations in α- or in β-tubulin. Note that a colcemid-resistant line previously isolated (9) is more sensitive to taxol than is the wild type. An explanation for this observation will be provided in the discussion.

In a similar experiment, the mutants were tested for increased resistance or sensitivity to colcemid, a microtubule-disrupting drug. Because taxol and colcemid have antagonistic actions and because several of our mutants require taxol for growth, we had to include taxol in these experiments. Fig. 7 shows the effects of colcemid and taxol on the growth of the wild-type parent cell line, the fully taxol-dependent β-tubulin mutant Tax 11-3, and the colcemid-resistant β-tubulin mutant Cmd 4. Without taxol in the medium, wild-type cells grow in colcemid at concentrations of up to 0.01 µg/ml. In the presence of 0.05 µg/ml taxol, the cells can grow in colcemid at up to 0.02 µg/ml. The addition of taxol beyond
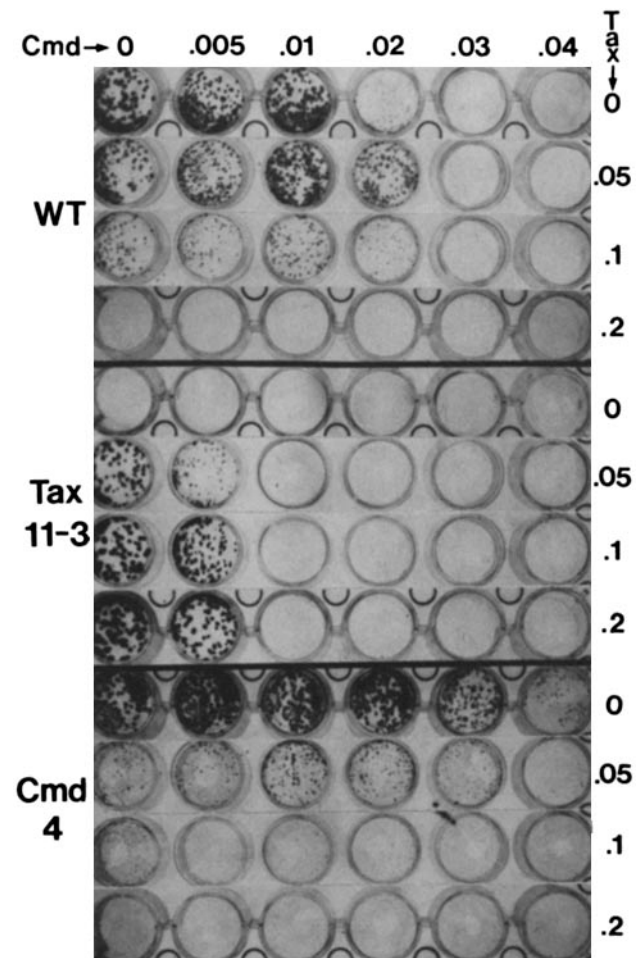

Figure 7. Growth of cells in various concentrations of colcemid and taxol. Plates were prepared and treated as described in legend to Fig. 6 except that colcemid and taxol were added in the concentrations shown. Colcemid concentrations in micrograms per milliliter increased from left to right and taxol in micrograms per milliliter increased from top to bottom for each strain. *WT*, wild-type parental cells; Tax 11-3, a fully taxol-dependent β-tubulin mutant; Cmd 4, a colcemid-resistant β-tubulin mutant.

0.05 μg/ml does not result in increased colcemid resistance and, in fact, inhibits the growth of the wild-type cells. As expected, mutant 11-3 did not grow without taxol, but its colcemid sensitivity could be measured as long as taxol was present in the growth medium. This cell line did not survive beyond 0.005 μg/ml colcemid regardless of how much taxol was added to the medium. Thus, mutant 11-3 is hypersensitive to colcemid as compared with wild-type cells. The addition of taxol to Cmd 4, a colcemid-resistant mutant, did not significantly alter its colcemid resistance but rather inhibited the growth of those cells at all concentrations tested. This result reinforces the data presented in Fig. 6 and indicates that the two drugs do not balance each other's effects in this cell line.

Because all of the taxol-resistant and -requiring mutants grew well in a concentration of 0.1 μg/ml of taxol, this concentration of the drug was included in all further experiments designed to test the colcemid sensitivity of the mutants. As indicated in Fig. 8, these mutants are all hypersensitive to colcemid except for Tax 1-19. The results did not change when the concentration of taxol was varied in the experiment (data not shown). The lack of colcemid hypersensitivity in Tax 1-19 is as yet unexplained but could indicate that this strain falls into a different class from the other taxol-resistant tubulin mutants we have isolated.

Taxol-dependent Tubulin Mutants Cannot Form a Functional Mitotic Spindle in the Absence of the Drug

As with the previously described mutant, Tax 18 (3), the mutants described in this paper require the presence of a microtubule-stabilizing agent for normal division and growth. To observe the disposition of microtubule arrays within the cells after taxol deprivation and also to determine if the tubulin alterations in these mutants affect their ability to form functional mitotic spindles, as was observed in mutant Tax 18 (10), mutant cells were grown with and without taxol and subjected to indirect immunofluorescence using antitubulin antibodies. When mutants 11-3 and 11-6 (as well as other
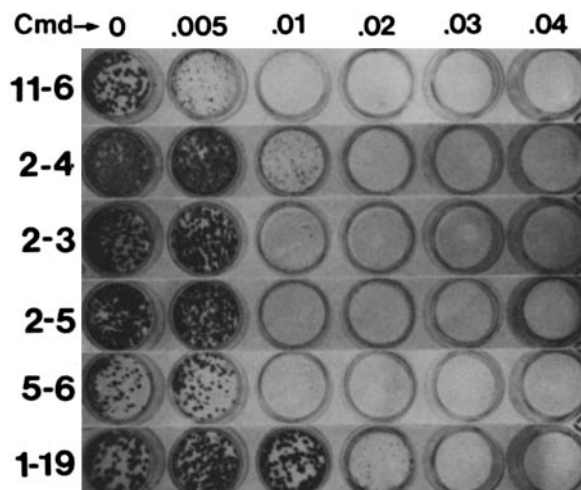

Figure 8. Growth of taxol-dependent and -resistant mutants in various concentrations of colcemid. Wells of 24-well dishes were prepared as described in legend to Fig. 6 except that the concentrations of colcemid in micrograms per milliliter shown at the top plus 0.1 μg/ml of taxol were added to each well. See text for details and legend to Fig. 6 for descriptions of mutant cell lines.

strains we have examined) were grown in the presence of taxol, the cells exhibited a normal cytoplasmic microtubule network similar in appearance to those of wild-type cells grown in the absence of taxol (data not shown). After 24 and 48 h of taxol deprivation, mutant cells were larger but still contained large arrays of microtubules as previously reported for Tax 18 (10). Thus, the taxol dependence in these tubulin mutants probably does not result from an inability of the cells to form cytoplasmic microtubules.

When mitotic cells were examined, spindles of the taxol-dependent cells grown in the presence of the drug (Fig. 9 a) appeared to be smaller than those of wild-type cells (Fig. 9 c). The polar areas of these cells fluoresced brightly, and bundles of microtubules (presumably kinetochore bundles) extended to the equatorial plane. Typical organization of chromosomes on the metaphase plate was confirmed using differential interference contrast microscopy (Fig. 9, b and d). Many anaphase and telophase cells and midbodies could also be seen in these preparations, indicating that these cells could complete mitosis. In mitotic cells of the mutants deprived of taxol for 24 h, a few spindles like the latter were observed, but most of the mitotic cells contained abnormal looking spindles with disorganized metaphase chromosomes (Fig. 9, e and f). After 48 h without the drug, mitotic cells were larger and contained more spindle poles and more disorganized chromosomes (Fig. 9, g and h). That no metaphase or anaphase spindles or midbodies could be found among cells grown in the absence of taxol indicates a lack of cell division.

Reversion of Mutant Tax 11-6 to Taxol Independence Is Associated with the Loss of the Altered α-Tubulin on Two-Dimensional Gels

Three of the mutant cell lines 2-4, 11-3, and 11-6 were subcloned, and spontaneous taxol-independent revertants were selected. The spontaneous reversion frequency of Tax 2-4 ($<3 \times 10^{-8}$) was much lower than those of the other two mutants (8.1×10^{-5} for 11-3 and 3.7×10^{-4} for 11-6), thus making isolation of revertants very difficult. However, several taxol-independent revertants of Tax 11-3 and Tax 11-6 have been isolated. Cells from 48 Tax 11-3 revertants and 18 Tax 11-6 revertants were grown, pulse-labeled with [^{35}S]methionine, and analyzed by two-dimensional gel electrophoresis. Based on this analysis, two types of taxol independent revertants of Tax 11-6 but only one type of Tax 11-3 revertant could be distinguished. 16 of the Tax 11-6 revertants still retained the altered α-tubulin spots on the gels. Two of the revertants, however, no longer contained the spots representing the altered α-tubulin. The co-segregation of taxol dependence and the altered tubulin at high frequency strongly suggests that the taxol-dependent phenotype in Tax 11-6 is a result of the alteration in α-tubulin. All of the 48 revertants of Tax 11-3, however, retained the altered β-tubulin. Thus, we cannot yet unambiguously ascribe taxol dependence in these cells to an alteration in β-tubulin, even though such an association is likely.

Discussion

A mutant of CHO cells that requires the presence of taxol, a microtubule-stabilizing drug, for normal growth and cell division was recently described by this laboratory (3, 10). When
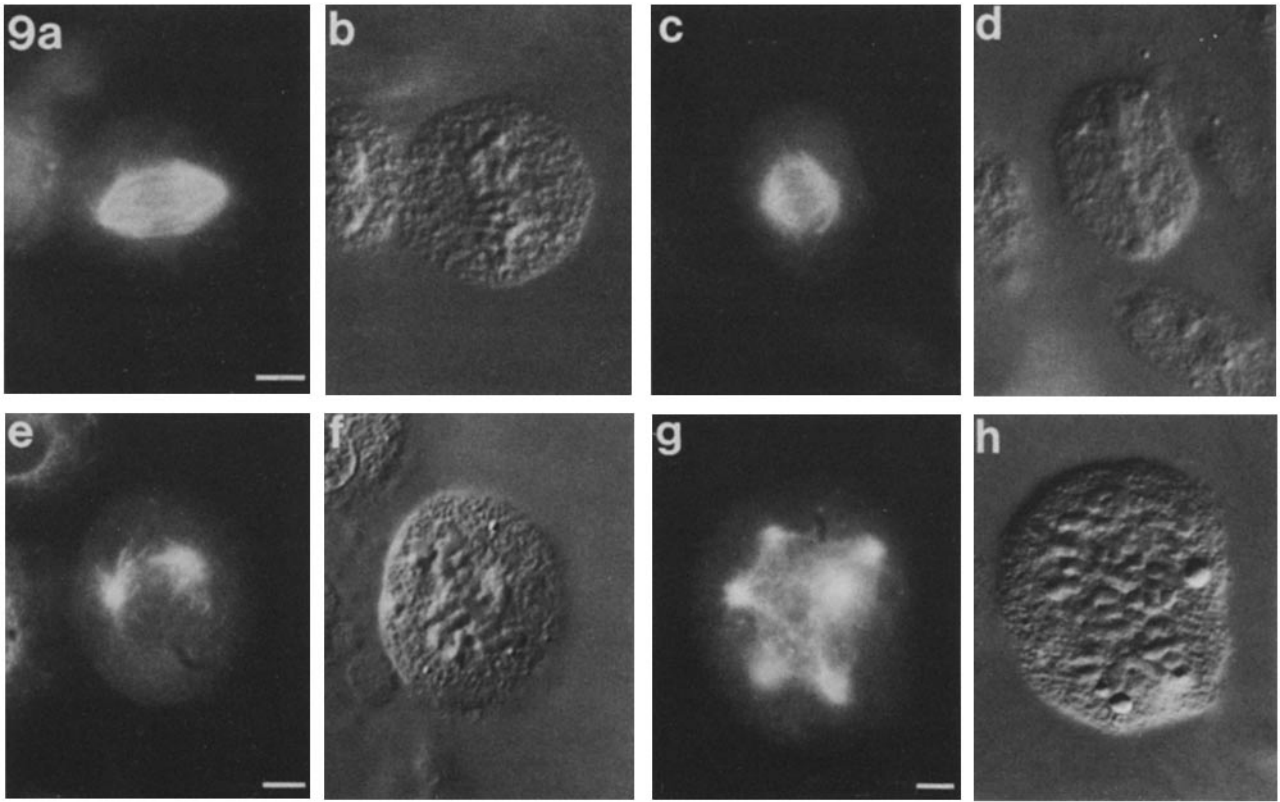
Figure 9. Antitubulin immunofluorescence and differential interference contrast (DIC) microscopy of CHO cells in mitosis. Cells grown on coverslips were fixed and stained for immunofluorescence as described in Materials and Methods. (*a* and *b*) Wild-type metaphase spindle; (*c* and *d*) metaphase spindle of Tax 11-6 grown in 0.2 μg/ml taxol. Note the small size of the spindle as compared with the wild type. (*e* and *f*) Bipolar cell of Tax 11-6 grown without taxol for 24 h. Note the concentration of fluorescence at the poles. (*g* and *h*) multipolar cell of Tax 11-6 grown without taxol for 48 h. Immunofluorescence is shown in *a*, *c*, *e*, *g*, the corresponding DIC images in *b*, *d*, *f*, *h*. Bars, 5 μm.

the mutant is deprived of the drug, the cells become larger and multinucleated and eventually die. Further studies indicated that the defect in the mutant cells affects their ability to form a functional mitotic spindle. Although the physiological consequences of the mutation in these cells have been characterized, the biochemical lesion responsible for the phenotype has not yet been determined.

We now report the isolation of many new taxol-resistant cell lines that are also taxol dependent. Of 139 taxol-resistant clones, 70 have a partial or complete requirement for taxol for normal growth and cell division. The physiological consequences of the lesions in the new taxol-dependent mutants are, for the most part, similar to those found in the previously described mutant, Tax 18 (3). The isolation and analysis of this new set of taxol-dependent mutants, however, has furthered our understanding of taxol dependence in several ways. First, in ~10% of the drug-dependent mutants, dependence correlates with and probably results from an alteration in a tubulin protein. Furthermore, reversion analysis of one mutant with an altered α-tubulin strongly suggests that mutations in α-tubulin can lead to taxol dependence, mutations in β-tubulin probably do as well. Finally, we have found that different mutants have varying sensitivities to taxol deprivation.

Only 10 of the taxol-resistant clones were cross-resistant to puromycin at a level that kills wild-type cells; none of the taxol-dependent clones was also puromycin resistant. Thus, in our selection, taxol-dependent clones were found at a

frequency of 50%, and putative permeability mutants comprised <10% of the drug-resistant clones. The selection for resistance to taxol, a microtubule-stabilizing agent, therefore, contrasts significantly with selections for resistance to microtubule-disrupting drugs (such as colcemid, vinblastine, and maytansine; see references 9 and 22) in that selections for resistance to the latter drugs results primarily in the isolation of permeability mutants with cross-resistance to puromycin.

Almost 10% of all the mutants isolated (13/139) and nearly 9% of the taxol-dependent subclass (6/70) contain altered forms of α- or β-tubulin detectable by two-dimensional gel analysis. The actual frequency of tubulin mutants among the taxol-resistant and -dependent mutants is probably higher because only those mutants that show an alteration in a tubulin protein in their two-dimensional gel patterns (i.e., in their isoelectric points) were identified. On a random basis, only one third of missense mutations cause a change in the isoelectric point of a resultant protein (28). Alterations in α- and β-tubulin were common among taxol-resistant cells, suggesting that such alterations may lead to resistance or dependence on taxol. The isolation of spontaneous revertants 11-6 that are no longer taxol dependent and have a wild-type two-dimensional gel pattern confirms this suggestion.

The extent to which taxol dependent mutants require the drug appears to vary. In general, taxol-dependent tubulin mutants behave like Tax 18, the mutant characterized earlier (3, 10). However, some clones have more or less stringent requirements for taxol. In our initial screening for taxol

dependence, we observed that some mutants, while still able to form viable colonies in the absence of taxol, do so at a much slower rate than taxol-independent clones. Furthermore, many aberrant cells, similar to those seen in mutants more stringently dependent on taxol, can be observed among these partially dependent cells when they are deprived of the drug. Growth curves show this distinction between partially and completely dependent cells even better. Without taxol, completely drug-dependent cells cease dividing altogether after 24 h (Fig. 3b). Partially dependent clones can still grow after removal of taxol from the medium, but the average doubling time becomes much greater (Fig. 3c).

All five new taxol-dependent mutants that we have examined by indirect immunofluorescence with antibodies against tubulin appear to be affected in their ability to assemble a functional mitotic spindle. In all of the mutants, there were large networks of cytoplasmic microtubules during interphase, even in those which had become quite large after 2 d of growth without taxol. Mitotic cells in cultures deprived of taxol had only very short spindle microtubules concentrated in the polar regions. In large mitotic cells, several of these polar microtubule remnants could be seen. Thus, in all mutants so far examined, the defect leading to taxol dependence results from an inability of the cell to polymerize properly its spindle microtubules into a functional spindle unless the drug is present.

When mutants were observed by phase microscopy after 1 or 2 d of taxol deprivation, most mutants assumed morphologies similar to those of Tax 18 cells deprived of the drug. Cells became progressively larger and were found to contain many micronuclei. Many of these cells appeared to die after 4–5 d of taxol deprivation. In one taxol-dependent β-tubulin mutant, Tax 2-4, the effect was even more drastic. After 24 h without the drug, many cells had died, and the culture became increasingly necrotic the longer the cells were deprived of taxol. This mutant appears to have a more stringent requirement for the drug than most taxol-dependent isolates.

To explain these observations, taxol dependence in these mutants may be thought of in terms of a simple model which we have recently presented (7, 22). In brief, if one imagines that microtubules in the cell must be maintained in a tightly regulated equilibrium between the polymerized (microtubules) and unpolymerized ($\alpha\beta$ heterodimers) states, then in the presence of a microtubule destabilizing drug (e.g., colcemid or vinblastine) this equilibrium will be shifted toward the unpolymerized state to such a degree that the cell will be unable to carry out microtubule-mediated events (e.g., mitosis). Conversely, a microtubule-stabilizing drug (taxol) will prevent the cell from depolymerizing its microtubules or reassembling them in an ordered array for functions such as mitosis. We can then think of a colcemid-resistant mutant as a cell line with an altered α- or β-tubulin that confers increased stability upon the microtubules. Because these microtubules are more stable, a higher concentration of colcemid would be needed for symptomatic depolymerization to occur. At the same time, however, less taxol would be needed to produce hyperstability of these microtubules and the consequent cell dysfunctions. Conversely, a taxol-resistant mutant would have less stable microtubules. Thus, a higher concentration of taxol would be required to produce microtubule hyperstability, but lower concentrations of colcemid would produce microtubule

disruption. A taxol-dependent mutant may then be thought of as an extreme case of a taxol-resistant cell, i.e., the genetic lesion has caused the microtubules to become so unstable that spindle microtubule assembly cannot occur in the absence of an exogenous stabilizing drug, taxol. This mechanism also predicts the existence of colcemid-dependent mutants, but to date only one has been isolated through gene transfer experiments using a strain that was already colcemid resistant as the source of the donor DNA (8). It is not surprising that colcemid-dependent mutants are more difficult to obtain, since a mutation to hyperstability of microtubules would require stronger subunit-subunit interactions than have already evolved in the wild-type strain. A mutation that produces hyperlabile microtubules as seen in taxol-dependent mutants, on the other hand, would result in less perfect subunit-subunit interactions. Such mutations should occur much more frequently.

Although few data are available from other systems to support this model, Warr et al. (30) have reported isolating CHO mutants with similar patterns of cross-resistance. Also, the properties of some tubulin mutants of *Aspergillus* are explained very well by it (12, 20). Ben A33, for example, is a temperature-sensitive (ts) β-tubulin mutant of *Aspergillus* (20). At the restrictive temperature these cells fail to exhibit nuclear movement or division even though they have apparently normal spindle microtubules by immunofluorescence (12). Interestingly, the ts phenotype can be suppressed by a variety of microtubule inhibitors or by tub A1, a mutation in α-tubulin (20). These results may be explained by postulating that the ben A33 mutation confers hyperstability on the microtubules and that it is the inability of the microtubules to depolymerize appropriately that leads to the mitotic defect. The addition of exogenous depolymerizing drugs or a mutation in α-tubulin resulting in hyperlabile microtubules could then compensate for the hyperstability introduced by the ben A33 mutation, leading to a microtubule population with approximately normal stability and function. Thus, these experimental observations can be easily interpreted based on the model we have presented to explain our own data.

The morphologies of our mutants after taxol deprivation resemble those of wild-type cells after exposure to microtubule destabilizing drugs. In the latter, cells also become large and syncytial. When considered in terms of our model, it can be seen that the states of the microtubules in both situations are essentially the same (i.e., shifted towards depolymerization), but one is the result of a genetic lesion and the other is drug induced. We may think of the present set of taxol-dependent tubulin mutants as all having highly unstable microtubules. However, since there are varying degrees of taxol dependence (Tax 2-3, 2-5 < Tax 18, Tax 11-3, 11-6 < Tax 2-4), there is probably a continuous spectrum of microtubule stability dictated by the strength of subunit-subunit interactions between tubulin subunits or between tubulin and microtubule-associated proteins. Mutations that affect these interactions can thus produce varying degrees of microtubule instability depending on the severity of the disruption of subunit-subunit interactions.

Mutations in tubulin genes may account for taxol dependence of several of our mutants, but in most of our new mutants, as in Tax 18, the biochemical lesion remains unknown. Taxol-dependent cells cannot be permeability mu-

tants because they require the drug. Because the phenotypes are similar among these different mutants, microtubules are probably the affected sites even in those mutants with no alteration in the electrophoretic mobility of tubulins. If alterations in the tubulin genes were the only ones responsible for the taxol-dependent phenotype, we would expect to be able to detect alterations in isoelectric points of tubulins in ~30% of our mutants on a random basis. However, only 10% contain detectable tubulin alterations as compared with 30–50% among colcemid- and maytansine-resistant mutants. Because microtubules are probably the affected structures in these mutants, a possible explanation is that the phenotype may result from alterations in microtubule-associated proteins. We are currently screening the taxol-dependent mutants for such alterations.

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