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GRANT NUMBER DAMD17-94-J-4352

TITLE: Paclitaxel (Taxol) Resistance in Breast Cancer Cells

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REPORT DATE: October 1996

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 1996	Annual (1 Oct 95 - 30 Sep 96)

4. TITLE AND SUBTITLE
Paclitaxel (Taxol) Resistance in Breast Cancer Cells

5. FUNDING NUMBERS
DAMD17-94-J-4352

6. AUTHOR(S)
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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Stanford University
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

Because breast cancer MDA 435 and MCF-7 cells do not grow clonally at single cell densities, we studied mechanisms of resistance to paclitaxel (Taxol) in single-step selected clones of human sarcoma cells. Fifteen populations of MES-SA cells were exposed to paclitaxel at 10 nM for 7 days. The mutation rate was 8 x 10^{-7} per cell generation. Analysis of variance supports a stochastic cell survival mechanism of spontaneous mutation rather than induction of an adaptive response under these selection conditions. Analysis of *mdr*1 gene expression by rt-PCR was positive in 4 of the 9 clones with stable resistance. Accumulation of paclitaxel was decreased in these but not in *mdr*1 -negative clones. High resistance to paclitaxel (36 to 93 fold) was selected in all 9 mutants. Those with *mdr*1 activation demonstrated cross-resistance to vinblastine, doxorubicin, and etoposide, whereas the other 5 mutants were cross-resistant only to vinca alkaloids. All surviving clones displayed reduced expression of the 5ß and the ß4 ß tubulin isotype transcripts in comparison with the parental cell line. The results suggest an important role for non-MDR mechanisms of resistance to paclitaxel. These mechanisms do not involve reduced drug accumulation, and provide cross-resistance among both paclitaxel and tubulin depolymerizing agents.

14. SUBJECT TERMS
Breast Cancer , drug resistance, paclitaxel, Taxol, tubulin, microtubules

15. NUMBER OF PAGES
28

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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2/10/97

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INTRODUCTION

Paclitaxel (Taxol) is a terpenoid agent extracted from the bark of the yew tree, *Taxus brevifolia*. The drug has displayed significant antitumor efficacy against breast and ovarian cancers in clinical trials (1-3) and is being investigated as an active agent in many other cancers including lung, head and neck, bladder, and lymphomas. Tubulin is the target molecule which mediates the cytotoxicity of paclitaxel (4). The best understood mechanism of resistance to paclitaxel *in vitro* is the multidrug resistant (MDR) phenotype mediated by the multidrug transporter P-glycoprotein (P-gp) (5). Other possible mechanisms of resistance to paclitaxel include alterations in microtubule composition and/or dynamics. Alterations in α- and ß-tubulin subunits have been described both in human and in non-human paclitaxel-resistant cell lines (6, 7).

In order to evaluate the frequency and the nature of these various mechanisms of resistance to paclitaxel, we have performed a Luria-Delbrück fluctuation analysis with the human sarcoma cell line MES-SA (8). The analysis is based on the variation that is observed in the emergence of surviving cell clones in parallel populations, after stringent selection by a 7 day drug exposure. Spontaneous mutation may be distinguished from induction of resistance by drug exposure under these experimental conditions. In contrast to prevalent cellular models of drug resistance derived by multiple step-wise selections, the surviving clones in fluctuation analysis have experienced a single drug exposure.

In these experiments, *mdr*1 expression was found to be positive by rt-PCR in 4 out of 9 resistant surviving clones (44%). Accumulation studies of labelled paclitaxel did not support an alternative efflux pump. Given the fact that the *mdr*1-negative clones displayed resistance not only to paclitaxel but also to the depolymerizing agents, we sought to determine whether alterations in the tubulin content and/or isotype profile existed in these clones. Our findings indicate that except for one clone, the total tubulin

content was not significantly altered in comparison to the parental cell line. All of the surviving clones, both sensitive and resistant (*mdr*1 positive and negative) expressed reduced RNA levels of ß4 and 5ß tubulin isotypes. These data suggest that the reduced ß tubulin isotype transcript levels were a consequence of stringent exposure to paclitaxel, and that other non-*mdr*1 mechanisms are implicated in resistance to the drug.

We chose to use the human sarcoma cell line MES-SA in this study instead of breast cancer cells because the breast cancer cell lines MDA 435 and MCF-7 do not grow clonally at single cell densities. By utilizing the sarcoma cells, we were able to determine mechanisms of resistance to paclitaxel (Taxol) in single cell clones. The information derived on tubulin expression will be applied to multistep selected, paclitaxel resistant breast cancer cell lines, which are currently being developed.

BODY

MATERIALS AND METHODS

Cells. The MES-SA cell line, which was derived in our laboratory from sarcomatous elements of a uterine mixed mullerian tumor, and its MDR variant MES-SA/Dx5, have previously been described (9, 10). The MES-SA cell line is pseudodiploid (45, XX) and has displayed relative karyotypic homogeneity over the past few years, as well as a stable drug-sensitivity phenotype. Monolayer cultures of MES-SA and its variants were grown as described, with a plating efficiency of 85% (10).

Drugs. Paclitaxel and etoposide were obtained from Bristol-Myers Squibb Co. (Evansville, Ind.), vinblastine and vincristine from Eli Lilly and Co. (Indianapolis, IN) and doxorubicin from Adria Laboratories (Columbus, OH). All other cytotoxic agents were obtained from the National Cancer Institute (Bethesda, MD). [^{3}H]-paclitaxel (19 Ci/mmol, 1 Ci/ml) was purchased from Moravek Biochemicals, Inc. (Brea, CA).

Fluctuation analysis experiment. Fifteen 25 cm^2 tissue culture flasks (Corning Glass Works, Corning, NY) were seeded with MES-SA cells at low density (2,000 cells per flask) and allowed to grow to near confluence (average of 2.7×10^6 cells per flask). The total cell populations from each flask were seeded into separate 96-well plates. The following day treatment with paclitaxel was begun at 10 nM. In parallel experiments with 25 nM and 50 nM paclitaxel, there were no surviving clones at 25 nM among 15 populations, and 2 colonies at 50 nM which were not stably drug resistant.

These selection conditions were chosen based on preliminary experiments demonstrating that the IC_{50} (drug concentration resulting in 50% inhibition of MTT dye formation) of MES-SA cells for paclitaxel is 1 nM, and to minimize the proportion of cells surviving on the basis of stochastic, epigenetic mechanisms. Drug-containing medium was changed every day over a period of 7 days, then replaced with drug-free medium. Surviving colonies were allowed to grow for another 2 to 3 weeks and

counted. Wells containing single colonies were harvested and expanded in drug-free medium for further studies.

A control experiment was performed to establish that the probability of pre-existing resistant clones in the original seeded populations was very low. Fifteen populations of 2,000 MES-SA cells were seeded into 96-well plates and treated directly with 10 nM paclitaxel under the same conditions as above, but without prior expansion of the populations.

In another control experiment designed to evaluate the spontaneous variation in the degree of expression of total tubulin and tubulin isotypes in MES-SA clones, clones of MES-SA were obtained by limiting dilution (0.1 cell/well), with no exposure to drug.

Analysis of mutation rates. Two methods were used to calculate the mutation rates: (1) the P_0 method of Luria and Delbrück (8):

$\mu = [(\ln 2)(-\ln P_0]/(N_t - N_0)$

where P_0 represents the fraction of cultures with no mutants, μ is the rate of amplification per cell generation, and N_t and N_0 are, respectively, the final and the initial cell number, adjusted for plating efficiency;

2) the method of Catcheside (11) according to the following equation:

$\mu = 2 \ln 2(r_2/N_2 - r_1/N_1)/g$

where μ is the mutation rate per cell generation, r represents the number of resistant colonies at a given time, N is the cell number adjusted for plating efficiency and g is the number of generations.

Growth inhibition assays. After expansion in drug-free media, surviving clones were harvested and assayed for *in vitro* drug sensitivity to paclitaxel, vinblastine, vincristine, doxorubicin and etoposide. Growth inhibition was evaluated by the MTT colorimetric assay in quadruplicate as previously described (12).

Amplimers used for reverse-transcriptase polymerase chain reaction (rt-PCR). The oligonucleotides used as amplimers in this study were synthesized by Operon

Technologies (Alameda, CA). Amplimers for *mdr*1 were the following: 5' 3020-3037; 3' 3168-3187. We designed the following primers for analysis of the tubulin isotypes (Arabic numerals refer to the gene, Roman numerals refer to the tubulin protein isotype class):

- Bα1 forward primer: 5' (1003,1020) ATC AAG ACC AAG CGT ACC 3'
- Bα1 reverse primer: 5' (1363,1380) CAG CAC CTT TGT GAC GTT 3'
- Kα1 forward primer: 5' (1000, 1017) ACC ATC AAA ACC AAG CGC 3'
- Kα1 reverse primer: 5' (1363, 1380) TGC AGG GCC AAA AGG AAT 3'
- Hα44 forward primer: 5' (139, 158) CCT TCA CCA CCT TCT TCT GT 3'
- Hα44 reverse primer: 5' (230, 149) TCG GTA TGG GCC ATT TCG GA 3'
- M40 (class I) forward primer: 5' (-42,-22) CCA TAC ATA CCT TGA GGC GA 3'
- M40 reverse primer : 5' (226,246) GCC AAA AGG ACC TGA GCG AA 3'
- ß9 (class II) forward primer : 5' (1131,1150) CGC ATC TCC GAG CAG TTC AC 3'
- ß9 (class II) reverse primer : 5' (1301,1319) TCG CCC TCC TCC TCC TCG A 3'
- ß4 (class III) forward primer: 5' (1,15) ATG AGG GAA ATC GTG 3'
- ß4 reverse primer: 5' (223,243) AAA GGC CCC TGA GCG GAC ACT 3'
- 5ß (class IVa) forward primer: 5' (-85,-68) TCT CCG CCG CAT CTT CCA 3'
- 5ß reverse primer: 5' (167,186) TCT GGG GAC ATA ATT TCC TC 3'
- ß2 (class IVb) forward primer: 5' (-42,-22) GTC TAC TTC CTC CTC TTC CC 3'
- ß2 reverse primer: 5' (291,300) GTT GTT CCC AGC ACC ACT CT 3'
- γ forward primer: 5' (1055, 1072) AGT TGG CCA ACT TCA TCC 3'
- γ reverse primer: 5' (1349, 1367) TGC CCC AGG AGA TGT AGT 3'

The isotype classification used is the one described by Sullivan (13). These primers were designed using published sequence data for M40, 5ß and ß2 isotypes (14) or, in the case of ß4 isotype a consensus forward primer and partial sequence information generously provided by Kevin Sullivan (Scripps Research Institute, La Jolla, CA). Primers for M40, 5ß, ß2 and ß4 were designed to span introns. In the case of class II

isotype, sequence was provided by screening expressed sequence tags from the EMBL GeneBank, using the peptide sequence previously reported by Cowan et al. (EST T03799) (15).

Reverse transcriptase polymerase chain reaction (rt-PCR). The isolation of total RNA and rt-PCR were performed as previously described, with annealing at 55 ^{o}C and extension at 72 ^{o}C (16). Given the caveats of semi-quantitative PCR, we have tested each sample over a range of different number of PCR cycles and at different concentrations of cDNA. Ribosomal cDNA was used as an internal control for standardization and comparison of samples. The amplimers used for ribosomal RNA were the following: 5' 1501-1520); 3' 1846-1826. cDNAs were first adjusted in order to provide ribosomal PCR products which differed by less than 10%. PCR samples were analyzed by 8% polyacrylamide gel electrophoresis, stained with ethidium bromide, and analyzed by densitometric reading of bands on an Alpha Innotech IS-1000 image analyzer (San Leandro, CA).

Analysis of P-glycoprotein by flow cytometry. Aliquots containing 2 x 10^6 trypsinized cells were washed and incubated with UIC2 monoclonal antibody (graciously provided by Drs. E. Mechetner and I. Roninson, U. Illinois, Chicago) or a mouse IgG2a isotype control at a final concentration of 40 μg/ml for 1 hour at 4 ^{o}C. Cells were then washed, incubated with a FITC-conjugated goat anti-mouse IgG2a secondary antibody (Caltag, San Francisco, CA), washed and analyzed by flow cytometry.

Measurement of cellular glutathione levels. Approximately 1 x 10^6 cells were harvested and resuspended in media. Cells were washed twice and incubated with or without monochlorobimane (MCB, 40 μl, 2.2 mg/ml) for 15 minutes then resuspended in cold paraformaldehyde for 5 minutes. The samples were analyzed by flow cytometry (17).

Accumulation of [^{3}H]-Paclitaxel. Cells were tryspinized, and 1 x 10^6 cells were seeded in 6-well plates in quadruplicate. After overnight incubation, drug-free medium was discarded and cells were incubated in medium containing [^{3}H]-Paclitaxel (10 μCi) at 37 ^{o}C for one hour. After washing with PBS, cells were lysed in 4% SDS, and aliquots were sampled for total protein content and scintillation counting. Cellular drug accumulation was standardized to total protein content.

Evaluation of total tubulin content by immunoblotting. Cell pellets were resuspended in lysis buffer containing Tris-HCl pH 6.80, MgCl2 1mM, 2 mM EGTA, and 0.2% Tween 20 and protease inhibitors (PMSF 1 mM, leupeptin 50 μg/ml, pepstatin 1 μg/ml, trypsin inhibitor 1 mg/ml and aprotinin 20 μg/ml (Sigma, St. Louis, MO)). Total protein was quantified by the Lowry assay and samples containing 50 and 100 μg of total cell protein were prepared in SDS and boiled, applied to a 12% polyacrylamide gel then blotted onto a Hybond-ECL nitrocellulose membrane (Amersham) using a Sartorius apparatus (18). The membrane was blocked with 5% evaporated milk containing buffer, then incubated 1 hour at room temperature with pan-ß monoclonal antibody (Sigma, 1:3000 dilution), washed three times, incubated in goat anti-mouse antibody, washed, incubated in streptavidin-biotin, washed and processed in ECL reagents. Phosphocellulose purified tubulin prepared from bovine brain (generously provided by M.A. Jordan) was used as a control. Preliminary experiments were performed to determine the amount of protein and the concentration of antibody yielding values of tubulin content in the linear range.

RESULTS

Selection of paclitaxel-resistant variants and estimation of mutation rates. The data presented in Table 1 show the number of surviving colonies in each of the plates of the fluctuation analysis.

Table 1. Fluctuation analysis of paclitaxel-resistant MES-SA variants

Plate	Cells seeded (10^6 cells)	Positive wells per plate	Colonies per plate
1	3.7	3	3
2	4.0	0	0
3	1.0	8	8
4	3.5	18	29
5	2.6	18	24
6	1.6	9	10
7	0.5	1	1
8	2.3	16	17
9	4.1	27	34
10	1.5	13	13
11	0.6	0	0
12	2.9	7	7
13	1.7	44	94
14	3.1	0	0
15	3.3	7	9
Total	37	171	249
Average	2.5	11	17
Variance	1.48	146	574

Colonies appeared in 12 out of 15 plates. The average number of colonies per plate was 17, with a variance of 574. The fact that the variance is much greater than the mean suggests the occurrence of spontaneous mutations, rather than induction, conferring resistance to paclitaxel in these clones. Similar experiments were performed with higher concentrations of paclitaxel. At 25 nM, no colonies were observed in 15 plates. At 50 nM, two colonies were observed in one plate, with no survivors in the other 14 populations. Clones from eleven of the plates exposed to 10 nM paclitaxel were successfully expanded and further characterized.

According to the P_0 method, the mutation rate for resistance to 10 nM paclitaxel was 5.8×10^{-7} per cell generation. The value obtained by the Catcheside method was quite comparable: 8.2×10^{-7} per cell generation.

In most cases more than one clone was isolated from each of the 11 expanded populations. Accounting for the likelihood that these duplicate clones are related to the same original mutation, a total of 11 separate mutants were available for further characterization. One clone from each population was selected for further analyses.

In the control experiment in which fifteen populations of 2,000 cells were directly exposed to paclitaxel under the same conditions but without prior expansion of the populations, no surviving colonies were observed.

Expression of the *mdr*1 gene in paclitaxel-resistant clones. Expression of the *mdr*1 gene was evaluated using rt-PCR. The parental MES-SA cell line and its P-gp-positive subline MES-SA/Dx5 were used as controls. Four out of the 9 stably resistant populations expressed the *mdr*1 gene. In the case of these *mdr*1 positive clones, the presence of P-gp protein was confirmed by flow cytometric analysis of cells stained with the anti-P-gp UIC2 monoclonal antibody (data not shown).

Drug sensitivity phenotype of paclitaxel-resistant variants. After expansion, clones were repeatedly tested for sensitivity to paclitaxel over a period of at least six months. Initial assays performed after the 2 month expansion of surviving clones

showed that 9 out of 11 populations of cells displayed significant resistance to paclitaxel ranging from 36 to 93-fold compared to parental MES-SA cells. (Table 2).

Table 2. Drug-sensitivity phenotype of paclitaxel-resistant MES-SA variants

	Drugs				
Clones	PAC	VBL	VCR	DOX	VP-16
*mdr*1(+), resistant					
4B11	36	14	18	14	10
7H4	76	9	12	2	31
8A5	67	12	7	15	23
10F4	60	24	18	9	18
*mdr*1(-), resistant					
1B11	93	10	7	1	1
5A9	62	12	16	1	1
6D2	70	23	9	2	1
9H12	38	9	12	1	1
13G2	83	7	10	2	1
drug sensitive					
3C2	2	1	2	2	2
12F9	1	1	3	1	2

Results are expressed as fold-resistance of clones in comparison to the parental cell line MES-SA. Fold-resistance was obtained by calculating the ratio IC_{50} clone/ IC_{50} MESSA. Values are average of three experiments. PAC: paclitaxel; VBL: vinblastine; VCR: vincristine; DOX: doxorubicin; VP-16: etoposide

The two sensitive clones represent either early revertants or stochastic survivors by epigenetic mechanisms. However, over a period of several months in the absence of

drug, this resistance phenotype was lost with reversion to IC_{50} values comparable to that of the parental MES-SA cell line (data not shown).

Analysis of sensitivity to other compounds showed that all resistant clones displayed some degree of cross-resistance to the tubulin depolymerizing agents vinblastine and vincristine, although to a lesser degree than to paclitaxel. In contrast *mdr*1-positive clones also demonstrated resistance to doxorubicin and etoposide, which are substrates for the P-gp efflux pump. *Mdr*1-negative clones displayed sensitivities to these agents comparable to those of the parental cell line. All the clones retained sensitivity to cisplatin, hydroxyurea and 4-OH-cyclophosphamide comparable to the parental MES-SA line (data not shown).

Three categories of surviving clones were defined: drug sensitive (clones 3C2 and 12F9), *mdr*1-positive drug resistant (4H3, 7H4, 8A5, 10F4) and *mdr*1-negative drug resistant (1B11, 5A9, 6D2, 9H12, 13H2).

Accumulation of [^{3}H]-paclitaxel. Analysis of ^{3}H-paclitaxel accumulation in *mdr*1-negative resistant clones demonstrated that there was no accumulation defect of paclitaxel in these cells. This result suggests that a drug efflux mechanism, permeability change or significant alteration of total intracellular drug binding were not responsible for resistance to paclitaxel in these clones. In contrast, clone 7H4 which expressed *mdr*1 manifested a decrease in paclitaxel accumulation similar to that observed with the positive control MDR cells, MES-SA/Dx5.

Glutathione content. It has been suggested that modulation of the intracellular level of glutathione by buthionine sulfoximine influences the level of resistance to paclitaxel (19). To determine whether altered levels of glutathione were involved in resistance to paclitaxel in our clones, we determined their total glutathione content by FACS analysis. The glutathione content of the *mdr*1-negative resistant clones was similar to that of the parental MES-SA, with values ranging between 93 and 106% of that of the parental MES-SA cells.

Tubulin content and isotype expression. There was no consistent alteration in total tubulin content, although one of the *mdr*1 negative clones (9H12) demonstrated less than half the total tubulin content of MES-SA cells and two *mdr*1 positive clones had increased content.

Tubulin isotypes share a considerable degree of homology at the amino acid level, but are sufficiently divergent at the nucleotide level to allow the design of specific primers. Moreover, 3' untranslated regions differ considerably between isotypes and were used to design primers whenever these data were available. In all cases, the PCR products had the expected size and were further confirmed to be isotype-specific by sequencing (Sequenase PCR Product Sequencing Kit, Cleveland, OH). All of the primers reported in this publication have been sequenced and unambiguous sequences obtained, which were identical to the previously reported sequences. In the case of the ß4 reverse primer, the amino acid sequence was deduced and compared to other available class III sequences (chicken cß4 (20) and murine mß6, provided by S.A. Lewis). The human ß4 PCR product was shown to contain the same isotype-specific amino acid differences as the class III described in mouse and chicken. Furthermore, to determine whether the rt-PCR procedure could detect intronless tubulin pseudogenes (21), the following separate experiments were performed as controls. RNA, with or without digestion by DNAse (MessageClean™, Genhunter Corp., Brookline, MA), was tested by rt-PCR, either directly or after reverse transcription. These experiments did not show the generation of PCR products with these primers, ruling out the presence of pseudogenes in the MES-SA cell line (data not shown).

Tubulin isotype levels in the surviving clones were normalized to ribosomal RNA expression and compared to those of the parental MES-SA cells. Given the variability intrinsic to PCR, these experiments were performed on three separate batches of cells, with multiple dilutions of cDNA and different numbers of cycles to ensure that the reaction was in the linear range of amplification. Two separate batches

of parental MES-SA cDNA were used as controls in all experiments. Isotype levels of clones of MES-SA obtained by limiting dilution were determined and showed median values which were similar to those of MES-SA, with standard deviations of 0.21 to 0.71. Among the tubulin isotypes examined, there was a consistent decrease of the ß4 (class III) and 5ß (Class IVa) isotypes in all the single-step clones. The other ß-tubulin isotypes (M40, ß9, ß2), the α isotypes (Kα1, Bα1 and Hα44) and γ tubulin were expressed at levels comparable to those of the parental MES-SA cells, as well as the MES-SA limiting dilution clones. Of note is the fact that the 5ß and the ß4 values observed are at the lower limits of the range observed among clones of MES-SA obtained by limiting dilution.

DISCUSSION

Resistance to anticancer drugs may develop through a variety of mechanisms including increased efflux of the chemotherapeutic agent, as is the case in the multidrug resistant phenotype mediated by P-gp, and modification of the target molecule of the drug. In the case of paclitaxel, expression of the *mdr*1 gene and alterations in tubulin content have been the most widely investigated mechanisms of resistance (5, 22). In order to evaluate the mutation rates and relative frequencies of various mechanisms in the development of resistance to paclitaxel, we have performed a Luria-Delbrück fluctuation analysis by exposing parallel populations of MES-SA sarcoma cells to a single exposure of paclitaxel (8). Our findings are summarized as follows: 1) the mutation rate for resistance to 10 nM paclitaxel is 5 to 8 x 10^{-7} per cell generation; 2) analysis of variance supports a mechanism of spontaneous mutation and drug selection rather than induction of survival mechanisms by drug exposure; 3) expression of the *mdr*1 gene was found in 44% of the stably resistant clones; 4) there was no modification of drug accumulation in the non-P-gp clones; 5) there was no modification in glutathione content in any of the clones examined; 6) all single-step mutants displayed decreased expression of ß4 and 5ß ß-tubulin isotypes, and one clone displayed markedly reduced total tubulin content.

Methodological considerations. Fluctuation analysis was initially designed for the analysis of mutations in bacteria, and has since been extensively applied to mammalian cells, in particular neoplastic cells (23). There are a number of points which should be considered in interpreting the results from these analyses in mammalian cells (24): 1) the number of pre-existent variants/mutants in the parental population should be negligible; 2) the selecting agent should not also be capable of inducing the phenotype under the conditions of exposure; 3) variants should fall into an all or none category, with no intermediate states; 4) there should be negligible back variation to the

wild type phenotype and 5) the rate of variation (mutation) should be constant over time. In the case of our experiments, we have demonstrated by exposing cells to paclitaxel with no prior expansion that there were no pre-existent variants in the parental population. Second, variations occur during the expansion phase in the absence of paclitaxel or any other eventual mutagenizing agent, paclitaxel being introduced only during the selection step. Third, variations are analyzed on an all or none basis, relative to the capacity of the clones to survive the stringent exposure to paclitaxel. The low rate of back variation and the constancy of the variation rate during the duration of the experiment are reasonable working hypotheses, but were not demonstrated formally in the scope of this study.

Mechanisms of resistance to paclitaxel. In the case of paclitaxel, the best described mechanism of resistance is the expression of the P-gp efflux pump, responsible for the MDR phenotype (5). In our experiment, 4 of the 9 stably resistant clones (44%), or 4 of the 11 surviving populations (36%) displayed overexpression of the *mdr*1 gene, with a classical cross-resistance to non-tubulin agents such as doxorubicin and etoposide. However levels of P-gp were not as high as those seen in the case of the MDR variant line MES-SA/Dx5, which was obtained by prolonged exposure to doxorubicin with multiple step-wise increased drug exposures (10). Thus expression of the *mdr*1 gene accounts for approximately half of the resistance mechanisms to paclitaxel in this setting.

Other possible mechanisms of resistance include altered accumulation of drug, due to a transporter other than P-gp, and altered intracellular distribution or metabolism of the drug. Accumulation studies with labeled paclitaxel did not show altered accumulation in *mdr*1-negative clones. It is thus unlikely that the resistance observed in our *mdr*1-negative clones is due to overexpression of a transporter other than P-gp. Altered metabolism or distribution of paclitaxel were not explored in this work. However, biotransformation of paclitaxel occurs predominantly by mixed

function oxidation in the liver, and has not been observed to occur in tumor cells. Moreover, the cross-resistance to vinca alkaloids argues against a paclitaxel-specific mechanism such as drug metabolism in the non-MDR clones.

It has been reported that the reduction of glutathione content by L-buthionine sulfoximine antagonizes the cytotoxicity of paclitaxel (19). This effect was not due to altered uptake of drug, and immunofluroescence did not show any alteration in the morphology of microtubules. It has been suggested that the tubulin subunits α and ß, which contain 12 and 8 cysteine residues respectively, might undergo increased oxidation of their sulfhydryl groups when cellular glutathione levels are reduced, thereby altering their ability to polymerize. In the case of our clones, no alterations of total cellular glutathione were observed, thus ruling out altered glutathione content as a mechanism of resistance to paclitaxel in these cells.

The taxanes are unique among tubulin targeted cytotoxins in that they bind to polymerized tubulin only (4), although the exact binding site has not yet been determined (25, 26). Several investigators have searched for alterations in tubulin in paclitaxel-resistant cell lines. Cabral et al. have reported altered microtubule content in Chinese hamster ovary (CHO) cells selected for resistance to and/or dependence on paclitaxel (27). Haber et al. have recently reported increased tubulin content in murine cells displaying a classical MDR phenotype (28). Attempts to correlate ratios of polymerized to soluble tubulin and levels of expression of specific isotypes to paclitaxel resistance have yielded conflicting results (22, 28-30).

Interactions between anti-tubulin drugs and microtubules. Analysis of the interactions between chemotherapeutic agents and microtubules are highly complex. Microtubules are dynamic polymers, and alterations in assembly/ disassembly dynamics play a key role in the antimitotic effects of depolymerizing agents, such as vinblastine, and stabilizing agents, such as paclitaxel (31). Thus, a number of mechanisms may be involved in the induction of resistance (or hypersensitivity) to

these agents: 1) reduced binding of the drug, although this seems to seldom occur in the case of paclitaxel (32); 2) reduced amounts of the target molecule, tubulin (30); and 3) altered dynamic properties of microtubules.

It has indeed been suggested that cells containing "hypostable" microtubules tend to be more resistant to stabilizing agents such as paclitaxel, and hypersensitive to depolymerizing agents such as colchicine or vincristine (33). This has been shown to be the case in CHO mutants (6) as well as in a human lung cancer cell line selected for resistance to paclitaxel (7). All of our drug resistant mutants displayed cross-resistance to vincristine and vinblastine. It is therefore unlikely that our clones contained microtubules with altered stability. The total tubulin content was not significantly modified in most of our clones. Finally, we have shown that there is no modification of the accumulation of total paclitaxel in our single-step variants. Definitive data on drug binding would require purification of microtubules from the resistant cells.

Human genes encoding α and ß tubulins constitute a multigene family of 15 to 20 members (34), several of which are pseudogenes (21). The role of the various isotypes of tubulin remains controversial. As reviewed by Luduena (35) and Raff (36), there are some specific examples in which alterations in the content of an isotype has dramatic consequences. It has also recently been shown that isotype composition regulates microtubule dynamics *in vitro* (37). However, functional differences between tubulin isotypes have not been identified which may be generalized to a variety of cell models. In particular, transfection experiments have demonstrated that the exogenous isotypes are incorporated in all microtubular structures present in the transfected cells (38). Total tubulin synthesis appears to be tightly regulated by a variety of mechanisms. Cleveland et al. have described an autoregulatory mechanism taking place at the translational level, involving the first four amino acids, which are common to all known isotypes (39). More recent data supports the notion that adequate folding of tubulin subunits requires appropriate chaperone molecules, which act as regulators of tubulin function *in*

vitro (40, 41). Little is known concerning the regulation of the synthesis, utilization or degradation of tubulin isotypes, although a report by Sisodia et al. suggests that isotype-specific regulation occurs (42).

We have designed and tested primers for a number of human tubulin isotypes and applied them to our variants. All of our single-step variants have decreased levels of the ß4 (class III) and the 5ß (class IVa) ß-tubulin isotypes, relatively to the parental cell line MES-SA. In the study performed by Schibler et al. (6), only 10% of the paclitaxel-resistant CHO mutants had permeability alterations, whereas a substantial number displayed alterations either in α or in ß tubulin expression as evidenced by 2-D gel electrophoresis. It is important to note that the decreases which we observed in our clones were comparable in the three categories of variants analyzed: sensitive cells, *mdr*1-negative cells, and *mdr*1-positive cells. These data suggest that the stringent selection conditions in our single-step experiment have selected cells with a particular tubulin isotype profile but that this profile is not in itself sufficient to account for resistance to paclitaxel. The specific design of the Lüria-Delbruck fluctuation analysis, yielding both sensitive and stably resistant clones, offers the unique opportunity to distinguish between characteristics which are simply associated, as are the drug-sensitivity phenotype and the tubulin isotype profile in our experiment, and those which may be causally related. However, the cross-resistance with vinca alkaloids in the non-MDR clones strongly suggests that altered microtubule function is responsible for resistance to paclitaxel in these mutants. A number of other microtubule alterations may be involved in the occurrence of resistance, such as altered polymerization status or modification in the levels of microtubule associated proteins which regulate microtubule dynamics.

We have previously performed two fluctuation analysis experiments with MES-SA cells and other P-gp-transport substrates, doxorubicin and etoposide (16, 43). In the case of doxorubicin, all surviving clones expressed P-gp and displayed the classical

MDR phenotype. In the case of the VP16-resistant cells however, none of the mutants involved activation of *mdr*1, and most expressed reduced amounts of topoisomerase II, the target molecule for this agent. In the present experiment using paclitaxel as the selecting agent, approximately half (44%) of the surviving drug-resistant clones expressed the *mdr*1 gene. It thus appears that various substrates of P-gp differ markedly in their propensity to select for *mdr*1-positive clones. Confirmation of these findings in the clinical setting may have important implications for strategies designed to prevent the emergence of resistant tumors.

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