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Altered Expression of Mβ2, the Class II β-Tubulin Isotype, in a Murine J774.2 Cell Line with a High Level of Taxol Resistance*

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A series of taxol- and taxotere-resistant J774.2 cell lines has been characterized with respect to altered expression of β-tubulin, the cellular target for these drugs. Vertebrates have six classes of β-tubulin isotypes, each displaying a distinct pattern of expression. Although the functional significance of multiple β-tubulins has not been fully defined, there is evidence that the individual isotypes contribute to differences in microtubule dynamics and drug binding. To determine if alterations in the expression of β-tubulin isotypes play a role in taxol resistance, a PCR-based methodology was developed that permits highly specific amplification of each of the six known murine β-tubulin isotypes. Two isotypes, Mβ5 and Mβ3, were expressed abundantly in the drug-sensitive parental J774.2 cells. Although expressed at an extremely low level in the parental cells, expression of the Mβ2 isotype was increased 21-fold (<0.005) in the cell line most resistant to taxol. These findings suggest that a cell can alter its relative tubulin isotype composition in response to an external stress and specifically imply that altered expression of Mβ2, the class II β-tubulin isotype, may contribute to the development of high resistance to taxol.

Taxol (1) has demonstrated encouraging antitumor activity particularly in ovarian and breast carcinomas (2). The drug is a potent inhibitor of cell replication whose action results in the formation of stable microtubule bundles (3, 4). Despite encouraging activity of taxol in human tumors, development of resistance to the drug presents a serious clinical problem. Resistance to a variety of cytotoxic drugs is known to be mediated by either overexpression or mutation of their target proteins (5–9) or overproduction of membrane transporters such as P-glycoprotein (10, 11). In lower eukaryotes, mutations in both α- and β-tubulin genes have been shown to confer resistance to a variety of antifungal agents and herbicides (12–14). In the few studies that have examined tubulin alterations in taxol-resistant mammalian cells, electrophoretic variants of both α- (15, 16) and β-tubulin (17) subunits have been observed.

Attempts to characterize either altered expression or mutations of specific tubulin genes in mammalian cells have been hindered by the presence of multiple tubulin genes (at least 6 α-tubulin and 6 β-tubulin genes) (18–20). Within a particular species, multiple β-tubulin genes encode highly conserved polypeptides with individual sequences typically diverging in 2–8% of approximately 450 residues (21). Most of the variation between isotypes occurs in the last 15 residues of the β-tubulin molecule and these isotype-defining regions are conserved completely across species (22). The classification system adopted by Sullivan and Cleveland (18, 22) is based upon these conserved sequences and is therefore applicable to all vertebrates so far studied. In mouse, the β-tubulin isotypes are classified as follows: class I, Mβ5; class II, Mβ2; class III, Mβ6; class IVa, Mβ4; class IVb, Mβ3; and class VI, Mβ1 (18, 20). Each isotype class has a unique pattern of tissue expression ranging from constitutive for classes I and IVb to highly restricted for the brain-specific (classes III and IVa) and the hematopoietic-specific (class VI) isotypes (23). Class II β-tubulin is expressed at low levels in a wide variety of tissues; however, the major site of expression is brain (22). The differential expression and high conservation of isotypes have raised the question of whether isotypes provide unique biochemical properties that result in functional differences in microtubules. Several groups using isotype-specific sera have shown that microtubule structures in several cultured cell lines are composed of all of the expressed isotypes (24, 25). In addition, transfection with exogenous isotypes not normally expressed in the recipient cell results in incorporation of these isotypes into the microtubule cytoskeleton (24, 26). In contrast, other studies have suggested a specific function for individual isotypes. For example, during differentiation of P19 (27) and PC12 (28) neuronal cells, microtubule structures are preferentially made up of specific isotypes. Moreover, *in vitro* studies using purified bovine brain tubulin have indicated that there are inherent functional differences among the isotype classes in terms of microtubule dynamics (29), assembly (30, 31) and drug binding (32). The functional differences among isotype classes raise the question of whether isotype composition of a cell could vary in response to taxol, a drug that interacts with microtubules.

We have identified one domain of the taxol binding site on β-tubulin as the N-terminal 31 amino acids of the polypeptide (33). Since mutations in the drug binding site could lead to altered drug binding and result in drug resistance to taxol, it was of considerable interest to sequence this region of the β-tubulin genes expressed in taxol-resistant cell populations. Isotype-specific oligonucleotide primer pairs, which span the region of the β-tubulin genes encoding the taxol-binding do-

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TABLE I
Oligonucleotide primers used for amplification and sequencing of β-tubulin isotypes

Gene	Site of forward primer	Nucleotide sequence		Product size
		Forward	Reverse	
				bp
Mβ1	5′-UTR	5′-TCTTGGTTCAGGCTAGG-3′	5′-CTGGAGAGCAGACGTCCCAC-3′	146
Mβ2	5′-UTR	5′-CAAGAGCCTCCACCCCTTCT-3′	5′-TCCTCTCCAGTGGCAAGTCA-3′	171
Mβ3	5′-UTR	5′-GCTGTTCCGCATGTCGCC-3′	5′-CGCTATCTCCGTGGTAAGTG-3′	145
Mβ4	5′-UTR	5′-TCCATCAGACGCCACCAG-3′	5′-GAGGTCACTGTCCCCATGA-3′	157
Mβ5	5′-UTR	5′-CGGTACCTACATTGGAACC-3′	5′-AGAGATTCGGTCCAGCTGC-3′	194
Mβ6	3′-UTR	5′-AAGACAAGCAGCATCGTGTC-3′	5′-GGCTAAAATGGGGAGGACAG-3′	164
β_2-Microglobulin	Coding region	5′-ATTCACCCCCACTGAGACTG-3′	5′-CTCGATCCCAGTAGACGGTC-3′	86

main, have been designed for use in polymerase chain reactions (PCRs).[1] Using a single pair of oligonucleotide primers per isotype, both the level of expression and the nucleotide sequence of each of the six known murine β-tubulin isotypes can be determined with great specificity. Moreover, we have successfully applied this methodology to murine cell lines selected *in vitro* for various levels of resistance to either taxol or the closely related compound taxotere. Our results indicate that expression of Mβ2, the class II β-tubulin isotype, is increased 21-fold in the most highly taxol-resistant cell line.

MATERIALS AND METHODS

Cell Lines and Cytotoxic Drugs—All cell lines were grown in Dulbecco's modified Eagle's medium containing 1% L-glutamine, 1% nonessential amino acids, and 1% penicillin-streptomycin (Life Technologies, Inc.). J774.2 cells were maintained in either 10 or 20% heat-inactivated horse serum, J7-T1-45 in 20% serum, and all other cell lines were maintained in 10% serum. All drug-resistant cells lines were derived from the mouse macrophage-like drug-sensitive cell line, J774.2, by stepwise increases in drug concentration and maintained as described previously (34, 35) in the following concentrations of drug: J7-T1-45, 45 μM taxol (Drug Development Branch, National Cancer Institute); J7-T3-1.6, 1.6 μM taxol; J7-T4-0.4, 0.4 μM taxol; and J7-Ter1-0.08, 0.08 μM taxotere (a gift from Rhône-Poulenc).

Growth Inhibition Assays—The ED_{50} of each cell line was determined using the CellTiter 96™ AQ_{ueous} nonradioactive cell proliferation assay (Promega Corp., G5430). Cells were resuspended in either drug-free medium or, in the case of J7-T1, in 45 μM taxol that is required for normal cell proliferation (34). 1–2 × 10^3 cells/well were added to a 96-well tissue culture plate and allowed to attach overnight. Following the addition of 50 μl of medium, a serial dilution of the drug to be tested was carried out. The cells were incubated for 72 h, and then 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium and phenazine methosulfate solutions were freshly mixed and added to the cells. After 1–2 h at 37 °C, plates were read at 490 nm on a Bio-Tek Instruments microplate reader, model EL320.

Analysis of Cytoplasmic Tubulin—Cells were scraped off plates, washed twice with cold phosphate-buffered saline and resuspended in 10 mM Tris-HCl (pH 7.5), 250 mM sucrose, and 0.1 mM phenylmethylsulfonyl fluoride. After a 10-min incubation on ice, cells were lysed with a prechilled Dounce homogenizer and centrifuged for 10 min at 2500 × *g*. The supernatant was removed and centrifuged in a Beckman A95 airfuge rotor at 100,000 × *g* for 30 min. Equal amounts of cytosolic protein (100 μg) from each cell line were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 9% gels. Following electroblot transfer of the polypeptides, nitrocellulose blots were probed with a monoclonal anti-α (Sigma, T-9026) or anti-β tubulin (Sigma, T-4026) antibody, as described previously (36) and visualized using ^{125}I labeled anti-mouse antibody (Amersham Corp., IM.131). Blots were quantitated on a Molecular Dynamics PhosphorImager, model 400E.

RNA Analysis by the Polymerase Chain Reaction (RNA-PCR)—Total RNA (20–50 μg), isolated from drug-sensitive and -resistant cell lines with TRI Reagent™ (37) (Molecular Research Center, Inc.), was treated with RNase-free DNase (Boehringer Mannheim) to remove contaminating genomic DNA. Treated RNA (1 μg) was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase and random hexanucleotide primers, as described by Noonan *et al.* (38). For subsequent verification of complete removal of genomic DNA, aliquots of each RNA sample were also subjected to mock reverse transcription, in the absence of reverse transcriptase. A cDNA amount representing 50 ng of RNA was subjected to PCR for 35 cycles in a final volume of 25 μl using 1 unit of Amplitaq polymerase (Perkin-Elmer). Following an initial denaturation of 3 min at 94 °C, each cycle consisted of 30 s at 94 °C, 30 s at 55 °C, and 90 s at 72 °C. To verify removal of genomic DNA, PCR amplification was initially performed on the relevant samples using intron-spanning primers specific for the murine glyceraldehyde 3′-phosphate dehydrogenase gene. The sequences of the forward and reverse glyceraldehyde-3′-phosphate dehydrogenase primers based on published sequence (39) were 5′-ACGGCAAATTCAACGGCAC-3′ and 5′-TAGTGGGGTCTCGCTCCTGG-3′, respectively.

Unless otherwise specified, target β-tubulin and control β_2-microglobulin gene sequences were co-amplified in the same reaction. The gene-specific oligonucleotide primers are listed in Table I along with the size of the PCR products. The primers for the Mβ1, Mβ3, Mβ4, and Mβ5 isotypes were derived from the published sequences of these genes (40) and were designed to amplify the entire region encoding amino acids 1–31 of the relevant β-tubulin polypeptide. The unpublished nucleotide sequences of the 5′ end of the Mβ2 isotype and the 3′ end of the Mβ6 isotype were kindly provided by Drs. N. Cowan and S. Lewis (Dept. of Biochemistry, New York University School of Medicine, New York). Primers specific for the murine β_2-microglobulin gene were derived from the published sequence (41). Specific amplification of individual β-tubulin isotypes using these primer pairs was confirmed with full-length cDNAs of each of the six cloned murine β-tubulin isotypes, generously provided by Drs. N. Cowan and S. Lewis.

Following PCR, aliquots (10 μl) were electrophoresed on 12% polyacrylamide gels, and bands were visualized by UV transillumination using ethidium bromide staining prior to photography. Densitometry was performed on photographic negatives, and the ratio between the target and control PCR products for each cDNA sample was determined by dividing the densitometric volume of the target electrophoretic band by that of the control band (42, 43). At least three independent RNA-PCR analyses were performed, and the mean densitometric PCR ratio for each set of analyses was determined. Differences between the PCR ratios of various cDNA samples for a given β-tubulin isotype were assessed by Student *t* tests using two-sided *p* values.

Northern Blot Analysis—Total RNA (10 μg) was separated on a 1.5% agarose gel containing formaldehyde and transferred to GeneScreen Plus nylon membrane (DuPont NEN). Blots were prehybridized in buffer containing 10% dextran sulfate, 1% SDS, 1 M NaCl, 50 mM Tris (pH 7.8), and salmon sperm DNA. An isotype-specific oligonucleotide probe, 5′-GCTGGAGGACAACAGAAGTTCACTAAGGGTGCACACTG-TA-3′, was designed based on the published sequence of the 3′-untranslated region (UTR) for the Mβ2 isotype (40). Blots were hybridized at 57 °C overnight with γ-^{32}P-end-labeled probe, washed in 2 × SSC for 20–30 min at room temperature followed by a second wash in 2 × SSC, 0.1% SDS at 57 °C for 30 min, and exposed to Kodak X-Omat film for 1–2 days. To control for variations in sample loading, blots were reprobed with a *Xenopus* histone H4 probe.

Sequencing of β-Tubulin Isotypes—For each β-tubulin isotype expressed in a given cell line, the PCR product was subjected to direct cycle sequencing (*fmol* DNA Sequencing System, Promega Corp.) with the same oligonucleotide primers used for amplification. Following PCR amplification, aliquots (25 μl) were electrophoresed on low melting temperature agarose, and the bands were cut from the gel and purified (PCR Wizard Preps™, Promega Corp.). Where a given β-tubulin isotype was expressed at very low levels, the amount of PCR product available for sequencing was increased by band-stabbing the product (44) and then reamplifying it with the same primers. The two oligonucleotide primers used for amplification were end-labeled with [γ-^{33}P]ATP prior to sequencing both strands of the PCR product.

[1] The abbreviations used are: PCR, polymerase chain reaction; UTR, untranslated region; MAP, microtubule-associated protein.

TABLE II
Drug sensitivity of J774.2 and resistant cell lines

Cell line	ED_{50}[a] (μM) Taxol	Taxotere	Vinblastine	Colchicine	Doxorubicin
Cells in 20% serum					
J774.2	0.021	0.0047	0.0134	0.046	0.074
J7-T1–45[b]	>45[c] (>2195)[d]	3.4 (723)	0.34 (25)	6.3 (138)	6.4 (86)
Cells in 10% serum					
J774.2	0.022	0.0028	0.0326	0.039	0.063
J7-T3–1.6	4.1 (186)	0.4 (141)	1.1 (33)	4.5 (114)	3.1 (49)
J7-T4–0.4	1.0 (46)	0.041 (14)	0.15 (5)	0.69 (17)	1.2 (18)
J7-Ter1–0.08	2.4 (109)	0.39 (135)	0.71 (21)	1.8 (45)	3.0 (47)

[a] ED_{50}, drug concentration that inhibits cell division by 50% after 72 h.
[b] Cells maintained in 45 μM taxol during cross-resistance experiments.
[c] Maximum solubility of drug in medium is 50 μM.
[d] Numbers in parentheses, ratio of ED_{50} for resistant cell line to that for J774.2.

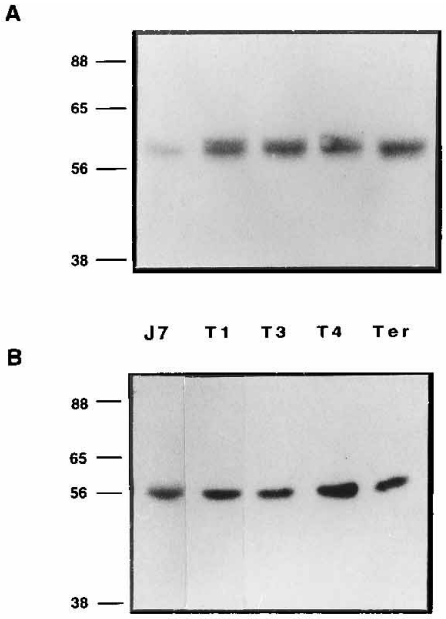
RESULTS

Cross-resistance of Cell Lines to Cytotoxic Drugs—The response of the taxol-resistant cell lines to a number of cytotoxic agents was tested using an MTT-based assay. Compared with the other drugs tested, the parental J774.2 cells were exceptionally sensitive to taxotere (Table II). The -fold resistance of each cell line for each drug was determined using the parental cells as a reference. The cell lines were most resistant to the selecting drug (taxol in the case of J7-T1, J7-T3, and J7-T4; taxotere in the case of J7-Ter1). However, there was significant cross-resistance to the other taxane, and all resistant cell lines demonstrated cross-resistance to colchicine, vinblastine, and doxorubicin.

Western Analysis of β-Tubulin in Drug-resistant Cell Lines—All taxane-resistant cell lines had increased levels of both α- and β-tubulin as compared with the parental cell line (Fig. 1). The relative level of α-tubulin was increased to a greater extent than that of β-tubulin. The increase in tubulin was independent of the level of taxane resistance in each of the cell lines. Thus, similar levels of β-tubulin were observed in the most taxol-resistant cell line, J7-T1, and in the least resistant cell line, J7-T4, despite the fact that they were maintained in taxol concentrations varying by 2 orders of magnitude. The lowest level of β-tubulin was detected in the taxotere-selected cell line, despite it being more resistant to both taxol and taxotere than J7-T4 cells.

RNA-PCR Analysis of Expression of Individual β-Tubulin Isotypes in Drug-resistant Cell Lines—The high level of sequence conservation within the coding regions of β-tubulin genes suggested that isotype-specific oligonucleotide primers should be designed within the nonconserved UTR of these genes (19). In view of the proximity of the gene sequence encoding a domain of the taxol-binding site (amino acids 1–31) to the 5′-UTR, isotype-specific primer pairs were therefore designed, where possible, so that the forward primer bound to the 5′-UTR of the relevant isotype and the reverse primer bound 3′ to the taxol-binding region (Table I). Since the 5′-UTR of the Mβ6 isotype has not been cloned,[2] primers specific for this isotype were designed entirely within the 3′-UTR of this gene. Absolute specificity of these primer pairs was confirmed by using cloned cDNAs encoding each of the six murine β-tubulin isotypes as individual templates (data not shown). The only exception involved amplification of an intense PCR product by the Mβ5-specific primers when using the cloned Mβ6 isotype as template. This resulted from a cloning artifact, whereby the cloned Mβ6 cDNA contained the complete Mβ6 coding region but juxtaposed with the Mβ5 5′-UTR due to failure to successfully clone the Mβ6 5′-UTR.[2]

[2] S. Lewis, personal communication.



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Relative Quantities

Cell Line	α Tubulin	β Tubulin
J774.2	1.0	1.0
J7-T1-45	2.62 ±0.80	1.97 ±0.52
J7-T3-1.6	2.35 ±0.17	1.70 ±0.33
J7-T4-0.4	2.92 ±0.25	1.95 ±0.10
J7-Ter-0.08	2.17 ±0.53	1.62 ±0.12

FIG. 1. **Analysis of tubulin content in taxane-resistant cell lines.** Immunoblot analysis of J774.2 (*J7*), J7-T1 (*T1*), J7-T3 (*T3*), J7-T4 (*T4*), and J7-Ter1 (*Ter*) using a monoclonal anti-α-tubulin antibody (*panel A*) or a monoclonal anti-β-tubulin antibody (*panel B*). A total of 100 μg of cytosolic protein was loaded onto each *lane* and visualized with an ^{125}I-secondary antibody. *Panel C*, relative quantities of tubulin in each cell line. J774.2 is given a value of 1.0. Each number represents an average of three independent experiments.

Having confirmed the isotype specificity of each of the β-tubulin primer pairs, expression of each isotype was determined in the J774.2 cell line and in each of the drug-resistant sublines. Since the tubulin multigene family is known to include a number of genomic, intronless, nonfunctional pseudogenes (19), complete absence of genomic DNA in the cDNA samples

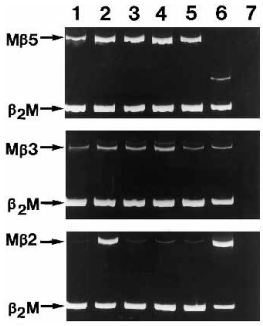

FIG. 2. **Expression of β-tubulin isotypes.** RNA-PCR analysis was performed in drug-sensitive murine macrophage J774.2 cells (*lane 1*), in taxol-selected J7-T1, J7-T3, and J7-T4 cell lines (*lanes 2–4*, respectively), and in the taxotere-selected J7-Ter1 cell line (*lane 5*). *Lane 6* contains genomic DNA isolated from J774.2 cells. Competitive RNA-PCR, involving co-amplification of the Mβ5, Mβ3, or Mβ2 β-tubulin isotype (*upper band*, as indicated) and β_2-microglobulin (*lower band*) gene sequences, was carried out as described under "Materials and Methods." *Lane 7*, water control.

for RNA-PCR analysis was a necessity. Therefore, RNA samples were treated exhaustively with DNase prior to reverse transcription, and complete removal of genomic DNA was confirmed by PCR using primers specific for the glyceraldehyde-3′-phosphate dehydrogenase gene, which is known to have multiple genomic pseudogenes (45).

Expression of each of the six β-tubulin isotypes was analyzed in the parental J774.2 cells and in the four drug-resistant sublines using a competitive PCR reaction involving co-amplification of a specific β-tubulin isotype with a control gene, β_2-microglobulin. The utility of this assay for semiquantitative analysis of expression of a range of genes, both in tumor samples and in cultured cells, has been previously demonstrated (42, 43). Following PCR, expression of the Mβ5, Mβ3, and Mβ2 isotypes was found in all five cell lines (Fig. 2). In contrast, PCR products were not detectable for Mβ1, Mβ4, or Mβ6 isotypes in any cell line following competitive PCR (data not shown). To determine whether low level expression of these isotypes would be detectable using a more sensitive assay, PCR analyses were repeated in the absence of the primers for β_2-microglobulin. Under these conditions, the Mβ4- but not the Mβ1- or Mβ6-specific primers produced a faint PCR product that was detectable at apparent equal intensity in all five cell lines (data not shown). To determine whether β-tubulin pseudogenes would be detectable by the isotype-specific primer pairs employed in this analysis, samples of genomic DNA isolated from the J774.2 cell line were amplified using each of the pairs of β-tubulin primers. A PCR product of the identical size to that amplified from cDNA samples was detected in genomic DNA following amplification with Mβ2- and Mβ3-specific primers (Fig. 2). These findings are consistent with the presence of β-tubulin pseudogenes homologous to Mβ2 and Mβ3.

Following densitometric analysis, the level of expression in each cell line of the three β-tubulin isotypes yielding detectable PCR products in the competitive RNA-PCR assay was quantitated relative to expression of β_2-microglobulin by determining the ratio between target and control PCR products for each sample (Table III). The mean PCR ratio for expression of the Mβ5 isotype in the J774.2 cell line was significantly greater ($p < 0.05$) than that of either the Mβ3 or Mβ2 isotype. The level of expression of the Mβ5 isotype was significantly higher in each of the four cell lines selected with either taxol or taxotere ($p < 0.005$ in each case) than in the parental J774.2 cells (Table III, Fig. 2). Despite its considerably higher resistance to taxol than that of the other three cell lines, the level of expression of Mβ5 in the J7-T1 cell line was not significantly different from that found in either the J7-T3 or J7-Ter1 cell line and in fact was significantly lower ($p < 0.05$) than the level observed in the least resistant J7-T4 cell line. A similar pattern of expression of the Mβ3 isotype was observed in the various cell lines (Table III, Fig. 2). Expression of the Mβ3 isotype, above the level in J774.2 cells, was observed in all four of the taxol/taxotere-selected cell lines, although this effect only achieved significance in the J7-T4 cell line. In contrast, a very different pattern of expression of the Mβ2 isotype was observed in these cell lines (Table III, Fig. 2). Expression of this isotype in J774.2, J7-T3, J7-T4, and J7-Ter1 cells lines was barely detectable, whereas high level expression of this isotype was observed in the extremely taxol-resistant J7-T1 cell line.

Northern Blot Analysis—To confirm the increased expression of the Mβ2 isotype observed using RNA-PCR in the highly taxol-resistant J7-T1 cell line, Northern blot analysis was performed. A 40-base pair synthetic oligonucleotide was designed based on the sequence of the 3′-UTR of Mβ2 (40). Isotype specificity of this probe was confirmed by Southern blot analysis using cloned full-length cDNAs of the murine β-tubulin isotypes (data not shown). Consistent with the RNA-PCR analysis, Mβ2 mRNA was clearly detectable in the J7-T1 cell line, whereas no Mβ2 expression was visible in J774.2 or the three other drug-resistant cell lines (Fig. 3).

Sequencing of the Taxol-binding Region—The complete nucleotide sequence encoding the first 31 amino acids of the β-tubulin polypeptide was determined for each of the four β-tubulin isotypes expressed in the J774.2 cells and in each of the taxol/taxotere-selected cell lines. Direct cycle sequencing of PCR products amplified by each of the isotype-specific primer pairs was performed using the same primers employed for PCR amplification. Comparison of the predicted amino acid sequences obtained for the Mβ2, Mβ3, Mβ4, and Mβ5 isotypes expressed in the J774.2 cell line with the sequences obtained for these isotypes expressed in normal mouse tissues (40)[3] revealed no alterations in any of the isotypes. At the nucleotide level, the sequences of the Mβ2 and Mβ4 isotypes in the J774.2 cells were identical to those obtained in normal mouse tissues. In both the Mβ3 and Mβ5 isotypes, minor differences were observed between the published sequences and those obtained in the J774.2 cell line. For the Mβ5 isotype, codon 23 in J774.2 cells, GTG, was altered from the published sequence of GTC. For the Mβ3 isotype, codon 9 in the J774.2 cells was found to be GCT compared with the published sequence of GCC, while codon 24, ATC, was altered from ATA. In all three cases the encoded amino acids, valine, alanine, and isoleucine, were unchanged (40). The sequence alterations detected in the parental J774.2 cells were preserved in each of the drug-resistant cell lines, and no mutations in the N-terminal 31 amino acids, a predicted taxol-binding region, were found in any of the taxol/taxotere-resistant cell populations.

DISCUSSION

While alterations in β-tubulin have previously been reported in mammalian cell lines resistant to the action of antimitotic agents (17, 46, 47), analysis has been limited primarily to the identification of changes in either electrophoretic mobility of tubulin molecules or in the total amount of β-tubulin mRNA or protein present in cells. Detailed analysis of the specific genetic changes, either qualitative or quantitative, affecting individual β-tubulin isotypes in such cell populations has not been described previously. The PCR-based methodology utilized in the present study provides a rapid and highly sensitive method for analyzing the expression of all known β-tubulin isotypes in a given cell population and for sequencing specific target regions

[3] N. Cowan and S. Lewis, personal communication.

TABLE III
Expression of β-tubulin isotypes in drug-sensitive and -resistant J774.2 murine macrophage cell lines

β-Tubulin isotype	Cell line				
	J774–2	J7-T1	J7-T3	J7-T4	J7-Ter1
Mβ5	0.44 ± 0.03[a]	0.82 ± 0.04[b] (1.86)[c]	0.91 ± 0.06[b] (2.07)	0.99 ± 0.04[b] (2.25)	0.73 ± 0.05[b] (1.66)
Mβ3	0.28 ± 0.05	0.37 ± 0.05 (1.32)	0.46 ± 0.11 (1.64)	0.60 ± 0.08[d] (2.14)	0.33 ± 0.05 (1.18)
Mβ2	0.04 ± 0.01	0.86 ± 0.04[b] (21.5)	0.04 ± 0.01 (1.0)	0.05 ± 0.02 (1.3)	0.04 ± 0.01 (1.0)

[a] RNA-PCR analysis was performed using primers to the β-tubulin isotype indicated, together with β_2-microglobulin control gene primers. Ratios between target and control gene products were determined densitometrically for each sample, and the data presented are the mean (±standard error) of at least three independent RNA-PCR analyses.
[b] The mean PCR ratio for the drug-resistant cell line is significantly greater ($p < 0.005$) than that of drug-sensitive J774.2 cells for a given β-tubulin isotype.
[c] Numbers in parentheses indicate the -fold increase in the mean densitometric PCR ratio of each drug-resistant cell line over that of the parent drug-sensitive J774.2 cell line for a given β-tubulin isotype.
[d] The mean PCR ratio for the drug-resistant cell line is significantly greater ($p < 0.05$) than that of drug-sensitive J774.2 cells for a given β-tubulin isotype.

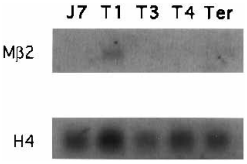

FIG. 3. **Northern blot analysis of Mβ2 expression in taxol-sensitive J774.2 (*J7*) and taxol-resistant cell lines (*T1, T3, T4,* and *Ter*).** 10-μg aliquots of total RNA from each cell line were electrophoretically separated and transferred to nylon membranes. Blots were hybridized with a 40-base pair oligonucleotide specific for the 3′-UTR of the Mβ2 gene (*upper panel*) and then rehybridized with a *Xenopus* histone H4 probe (*lower panel*) to control for variations in loading.

of each of the isotypes without the need for cloning of individual genes. The pairs of oligonucleotide primers utilized in the present analysis were highly specific for the individual β-tubulin isotype for which they were designed. This was confirmed not only by the use of plasmids containing each of the individual cloned β-tubulin genes but also by sequencing of the amplified PCR products.

The pattern of expression of β-tubulin isotypes in the J774.2 cell line, as determined by RNA-PCR, is consistent with previous results obtained in cultured mammalian cell lines, using either Northern analysis or isotype-specific sera. The Mβ3 and Mβ5 genes were the two most highly expressed isotypes in the J774.2 cells. Their homologues were found also to be expressed at high levels in human HeLa (24, 48), chicken embryo fibroblast, mouse 3T3, monkey CV1 (25), and Chinese hamster ovary cells (49). The Mβ2 isotype, which is detectable only at low levels in the J774.2 cells using the competitive RNA-PCR assay, has been shown to be predominantly expressed in cells of neuronal origin (22, 50). The minimal level of Mβ4 in J774.2 cells is consistent with the absence of this isotype in any normal tissue other than brain as determined by Northern analysis (50, 51). Finally, the two isotypes, Mβ1 and Mβ6, which were not detectable using even the most sensitive RNA-PCR assay, have been shown to be highly restricted in their expression (20).

Increased expression of β-tubulin was observed in all four of the taxol/taxotere-selected cell lines as determined both by RNA-PCR analysis and Western blotting. The latter method also detected an increased expression of α-tubulin that may reflect coordinate regulation of the intracellular levels of α- and β-tubulin as has been suggested previously (52). Alternatively, it may be that either qualitative or quantitative alterations in α-tubulin can in their own right contribute to the taxol-resistant phenotype. Cabral and colleagues (15, 17, 53) have reported several electrophoretic variants of α-tubulin in taxol-resistant cells, and Ohta *et al.* (16) have also reported variant electrophoretic mobility plus acetylation of α-tubulin in human taxol-resistant cells.

By Western analysis, there was no clear relationship between the level of β-tubulin and the level of resistance to taxol. Results entirely consistent with Western analysis were obtained by RNA-PCR for expression of the most abundant isotype, Mβ5, in the drug-resistant cell lines, and also, to a lesser extent, for expression of the Mβ3 isotype. In contrast to the present findings, Ohta *et al.* (16) and Minotti *et al.* (53) reported no significant increase in the total tubulin content of their taxol-resistant cell populations. However, the level of resistance in those cell lines (less than 10-fold) was far lower than in any of the cell lines described here. The present finding of increased tubulin in cells chronically exposed to taxol is consistent with reports of increased levels of both tubulin mRNA and protein in cells given a single brief exposure to the drug (54). Such increased synthesis of tubulin following taxol treatment appears to be an autoregulatory response documented in almost all animal cells (55). The ability of taxol to enhance the polymerization of tubulin subunits to form microtubules results in lower intracellular levels of unassembled α-, β-heterodimers, which in turn, stimulate the synthesis of tubulin. The fact that levels of β-tubulin in the relatively low level resistant J7-T4 cell line are as high as those observed in highly resistant J7-T1 cells is consistent with the concept that intracellular levels of tubulin are tightly regulated. Large increases in tubulin levels may be detrimental to the cell (52). Thus, a modest increase in total tubulin concentration may represent a physiological response of cells to taxanes.

The four taxane-resistant cell lines used in this study displayed a pattern of cross-resistance that is consistent with the multidrug resistance phenotype (34). The J7-T1 cell line has been used extensively for studies on P-glycoprotein (35), the energy-dependent drug efflux pump responsible for the multidrug resistance phenotype, and J7-T3, J7.T4, and J7.Ter1 cells also overproduce P-glycoprotein.[4] Bhalla *et al.* (56) have recently described highly taxol-resistant human cell lines that similarly overexpress P-glycoprotein. Taxol-resistant cells that do not overexpress P-glycoprotein tend to be of lower resistance and are frequently associated with hypersensitivity to drugs, such as colchicine, that destabilize microtubules (17). Such cells may also have a partial or complete dependence on taxol for normal cell growth and replication (16, 17), and this dependence has been described as a specific characteristic of cells with altered tubulin. The J7-T1 cell line is partially dependent on taxol for normal growth, with altered cell morphology and cell death ensuing following taxol withdrawal (34).[5] Taxol resistance in J7-T1 cells may result from an overproduction of P-glycoprotein and an increased expression of a single isotype of β-tubulin, Mβ2. It is not unusual for cells exposed to cyto-

[4] S. Horwitz, unpublished observations.
[5] S. Rao and S. Horwitz, unpublished observations.

toxic drugs to undergo multiple cellular changes.

Consistent with its characterization as primarily a neuronal β-tubulin isotype (22), Mβ2 expression barely was detectable in the parental J774.2 cells. Its dramatic up-regulation in the J7-T1 cell line suggests that this isotype may play a role in the high level of taxol resistance displayed by these cells. Cowan and colleagues (24, 40, 50) have suggested that different tubulin isotypes may bind distinct microtubule-associated proteins (MAPs), thereby contributing to the diversity of microtubule function. The pattern of expression of the Mβ2 isotype in mouse brain has a distribution indistinguishable from that reported for MAP3 (51), one of the few known situations where there is completely coordinate expression of a given MAP with a particular β-tubulin isotype. It is conceivable that increased expression of the Mβ2 isotype could alter the microtubule array by modifying the pattern of associated MAPs, making the cell more resistant to the stabilizing effects of taxol. It also has been demonstrated that transformation of drug-sensitive cells with DNA from colcemid-resistant Chinese hamster ovary cells containing a mutant β-tubulin gene results in a drug-resistant phenotype (57). No mutations were present in the first 100 nucleotides of the coding sequences, which include one domain of the taxol-binding site, for each of the expressed isotypes. This is consistent with the findings of Cabral and colleagues (17, 53), who failed to find evidence of altered drug binding in a number of taxol-resistant Chinese hamster ovary cell populations. Thus, despite the frequency with which mutations in tubulin that result in decreased binding affinity for antimitotic drugs are seen in lower eukaryotes (12, 13), it appears that this mechanism of drug resistance may be uncommon in mammalian cells. It has been proposed (17, 53) that the mutations in tubulin that occur in mammalian cells resistant to antimitotic agents are likely to lead to altered stability of the microtubules rather than mutations of the drug-binding site. Considering that taxol stabilizes microtubules, cells maintained in taxol may select for tubulin mutations that form less stable microtubules. Such microtubules actually may require taxol to function normally, and therefore cells containing the mutant tubulin become dependent on the drug.

Banerjee *et al.* (31) have raised the possibility that the assembly properties of a given preparation of tubulin molecules may be influenced by its isotypic composition and that the structural differences among tubulin isotypes could have functional significance. Indeed, against a plethora of evidence that the multiple β-tubulin isotypes may be used interchangeably (24, 25, 58), one of the few examples of selective utilization of specific β-tubulin isotypes involves the demonstration that the wild-type Mβ2 isotype is polymerized more efficiently than other isotypes (59) and is preferentially incorporated into stable microtubules (27) during neuronal differentiation. It is not clear that the association between the Mβ2 isotype and microtubule stability is present in cells other than those of neuronal origin (27). Nevertheless, the finding of increased expression of a β-tubulin isotype in taxol-resistant cells that is associated with tubulin polymerization and microtubule stabilization is puzzling in light of the idea that the presence of less rather than more stable microtubules in taxol-resistant cells might be expected. Clearly, the question of whether mutations will be found in the J7-T1 Mβ2 isotype at sites other than those sequenced so far and whether such mutations, if present, affect the stability of microtubules assembled using this isotype will be of considerable interest.

There are clearly a number of ways in which cells and human tumors can become resistant to the action of taxol, and a single resistant cell line may incorporate more than one mechanism of resistance. Since taxol has shown efficacy in some drug-refractory human tumors that were previously treated with drugs capable of selecting for and/or inducing P-glycoprotein, the question has arisen as to the most significant form of taxol resistance. The altered expression of the class II major neuronal isotype, Mβ2, may contribute to the overall resistance of the taxol-resistant murine cell line, J7-T1. Clarification of the precise ways in which β-tubulin is modified either in structure or expression in response to the drug has ramifications both for the design and synthesis of new taxol analogues and for strategies to overcome the development of taxol resistance in human tumors. The PCR-based methodology utilized in the present studies provides a valuable tool for investigating changes in taxol-resistant cells and human tumors and should have wide applicability in investigating the response of mammalian cells to the action of chemotherapeutic drugs that act in the tubulin/microtubule system.

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