Drug Resistance Patterns of Human Neuroblastoma Cell Lines Derived from Patients at Different Phases of Therapy

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ABSTRACT

To determine whether neuroblastomas acquire a sustained drug-resistant phenotype from exposure to chemotherapeutic agents given to patients in vivo, we studied neuroblastoma cell lines established at different points of therapy: six at diagnosis before therapy (DX), six at progressive disease during induction therapy (PD-Ind), and five at relapse after intensive chemoradiotherapy and bone marrow transplantation (PD-BMT). Cells were maintained in the absence of drug selective pressure. Dose–response curves of melphalan, cisplatin, carboplatin, doxorubicin, and etoposide for the cell line panel were determined by measuring cytotoxicity with a 96-well-plate digital imaging microscopy (DISMII) microarray. Drug resistance of cell lines progressively increased with the intensity of therapy delivered in vivo. The greatest resistance was seen in PD-BMT cell lines: IC50 values in PD-BMT cell lines were higher than clinically achievable drug levels by 1–37 times for melphalan, 1–9 times for carboplatin, 25–78 times for cisplatin, 6–719 times for doxorubicin, and 3–52 times for etoposide. Genomic amplification of MYCN did not correlate with resistance. Cross-resistance by Pearson correlation (r ≥ 0.6) was observed between: (a) cisplatin + doxorubicin; (b) carboplatin + cisplatin, etoposide, or melphalan; (c) etoposide + cisplatin, melphalan, or doxorubicin. These data indicate that during therapy, neuroblastomas can acquire resistance to cytotoxic drugs because of the population expansion of tumor cells possessing stable genetic or epigenetic alterations that confer resistance.

INTRODUCTION

Neuroblastoma is a malignant childhood neoplasm of the sympathetic nervous system. Myeloablative chemoradiotherapy supported with BMT1 improves survival for high-risk neuroblastoma patients (1, 2), especially if followed by treatment with high-dose 13-cis-retinoic acid (3). Even with intensive therapy, the majority of high-risk neuroblastoma patients eventually develop progressive disease that is refractory to further therapy (4). Drug resistance of neuroblastomas that progress during or after chemotherapy could depend on the altered expression of drug resistance genes (5–7), tumor cells in “sanctuary” sites of low-drug penetration (8), tumor hypoxia (9, 10), altered expression of drug resistance genes (5-7), tumor cells in that progress during or after chemotherapy could depend on the refractory to further therapy (4). Drug resistance of neuroblastomas that progress during or after chemotherapy could depend on the altered expression of drug resistance genes (5–7), tumor cells in “sanctuary” sites of low-drug penetration (8), tumor hypoxia (9, 10), tumor cells resting out of cell cycle (11), or all of the above. No current experimental data favors any of the above mechanisms. However, because most newly diagnosed high-risk neuroblastomas respond to chemotherapy whereas most relapsed tumors do not, it is likely that selection of tumor cells with alterations conferring drug resistance occurs.

Genomic amplification of MYCN is associated with advanced disease (12) and poor prognosis of neuroblastoma patients (13), but very little experimental data exist on the correlation of MYCN with chemotherapy sensitivity/resistance. It is not clear whether MYCN has a role in the emergence of drug resistance after initially successful chemotherapy, or whether the poor outcome of neuroblastoma patients with MYCN amplification is mediated by other mechanisms.

To develop a model system for identifying drugs that are effective against resistant neuroblastoma and for understanding the mechanisms by which neuroblastoma cells escape chemotherapy (especially myeloablative therapy), we have established a panel of cell lines from patients with neuroblastoma at various points during the course of their disease. Cell lines were established at diagnosis, at progressive disease during induction therapy, and at relapse after intensive chemoradiotherapy and BMT. The panel includes pairs of cell lines established from tumors of four patients at diagnosis and again at disease progression during induction chemotherapy. In this study, we have determined the sensitivity of this cell line panel to drugs commonly used in the therapy of neuroblastoma and the relationship of MYCN genomic amplification to drug resistance.

MATERIALS AND METHODS

Cell Lines. A panel of 17 human neuroblastoma cell lines were established from patients at various points during therapy: 6 DX, 6 PD-Ind, and 5 PD-BMT (3 PD-Auto-BMT and 2 PD-Allo-BMT). DX cell lines were derived from patients before any chemotherapy was given, PD-Ind cell lines came from patients treated with induction chemotherapy, and PD-BMT cell lines were obtained from patients treated with myeloablative chemoradiotherapy (Table 1).

Human neuroblastoma cell lines SMS-KAN, SMS-KANR, SMS-KCN, SMS-KCNR, SK-N-Be(1), SK-N-Be(2), SMS-SAN, SMS-LHN, and LA-N-6 have been characterized previously (14–16). CHLA-15, CHLA-42, CHLA-20, CHLA-15, CHLA-42, CHLA-20, CHLA-51, CHLA-8, CHLA-99, and CHLA-134 are newly established cell lines. All of the cell lines were cultured in complete medium consisting of Iscove’s modified Dulbecco’s medium (IMDM, Bio Whittaker, Walkersville, MD) supplemented with 3 mm l-glutamine (Gemini Bioproducts, Inc., Calabasas, CA), insulin and transferrin 5 μg/ml each and 5 ng/ml of selenium acid (ITS Culture Supplement, Collaborative Biomedical Products, Bedford, MA), and 20% heat-inactivated fetal calf serum (PCS). All of the cell lines used were studied within passages 10 through <30 (except SMS-KCNR, which was tested at passage 32, and SMS-SAN, which was tested at passage 40) and cultured at 37°C in a humidified incubator containing 95% air + 5% CO2 atmosphere. Cells were cultured without antibiotics to facilitate detection of mycoplasma, for which all of the cell lines tested negative. Cell lines were not selected for resistance in vitro.

Drugs and Chemicals. Melphalan (L-PAM), cisplatin (CDDP), carboplatin (CBDDA), and doxorubicin hydrochloride (DOX) were obtained from NIH (Bethesda, MD). Etoposide (ETOPO) was obtained from Bristol-Myers Squibb Co. (Princeton, NJ). FDA was purchased from Eastman Kodak Company (Rochester, NY), eosin Y from Sigma Chemical Co. (St. Louis, MO), and TRizol reagent from Life Technologies (Grand Island, NY).
Table 1 Characteristics of patients whose tumors gave rise to human neuroblastoma cell lines used in this study

<table>
<thead>
<tr>
<th>Phase of therapy and cell line</th>
<th>Therapy given to the patients</th>
<th>Site of specimen</th>
<th>Age at DX</th>
</tr>
</thead>
<tbody>
<tr>
<td>DX</td>
<td></td>
<td>Tumor</td>
<td>3 yr</td>
</tr>
<tr>
<td>SMS-KAN</td>
<td>none</td>
<td>Bone marrow</td>
<td>3 yr</td>
</tr>
<tr>
<td>SMS-KCN</td>
<td>none</td>
<td>Bone marrow</td>
<td>11 mo</td>
</tr>
<tr>
<td>SK-N-BE(1)</td>
<td>none</td>
<td>Bone marrow</td>
<td>2 yr</td>
</tr>
<tr>
<td>CHLA-15</td>
<td>none</td>
<td>Bone marrow</td>
<td>18 mo</td>
</tr>
<tr>
<td>CHLA-20</td>
<td>none</td>
<td>Bone marrow</td>
<td>3 yr</td>
</tr>
<tr>
<td>CHLA-42</td>
<td>none</td>
<td>Bone marrow</td>
<td>13 mo</td>
</tr>
<tr>
<td>PD-Ind</td>
<td></td>
<td>Tumor</td>
<td>3 yr</td>
</tr>
<tr>
<td>SMS-KANR</td>
<td>CTX&lt;sup&gt;a&lt;/sup&gt;</td>
<td>RAD</td>
<td>3 yr</td>
</tr>
<tr>
<td>SMS-KCNR</td>
<td>CTX DOX</td>
<td>RAD</td>
<td>11 mo</td>
</tr>
<tr>
<td>SK-N-BE(2)</td>
<td>CTX DOX</td>
<td>RAD</td>
<td>2 yr</td>
</tr>
<tr>
<td>CHLA-20</td>
<td>DOX</td>
<td>VCR</td>
<td>18 mo</td>
</tr>
<tr>
<td>SMS-LHN</td>
<td>CDP DOX</td>
<td>VCR</td>
<td>2 yr</td>
</tr>
<tr>
<td>LA-N-6</td>
<td>DOX</td>
<td>DTIC</td>
<td>5 yr</td>
</tr>
<tr>
<td>PD-Allo-BMT</td>
<td>CHLA-8</td>
<td>DOX CDDP</td>
<td>14 mo</td>
</tr>
<tr>
<td></td>
<td>CTX DOX</td>
<td>DOX ETOPO VCR</td>
<td>14 mo</td>
</tr>
<tr>
<td></td>
<td>CTX DOX</td>
<td>L-PAM MTX DTIC</td>
<td>14 mo</td>
</tr>
<tr>
<td>PD-Auto-BMT</td>
<td>CHLA-79</td>
<td>DOX</td>
<td>2 yr</td>
</tr>
<tr>
<td></td>
<td>CTX DOX</td>
<td>L-PAM</td>
<td>5 yr</td>
</tr>
<tr>
<td></td>
<td>CTX DOX</td>
<td>L-PAM</td>
<td>8.5 yr</td>
</tr>
<tr>
<td></td>
<td>CTX DOX</td>
<td>L-PAM</td>
<td>3 yr</td>
</tr>
<tr>
<td>VAX</td>
<td>CHLA-134</td>
<td>DOX CDDP CBDC</td>
<td>14 mo</td>
</tr>
<tr>
<td></td>
<td>CTX DOX</td>
<td>CDDP</td>
<td>14 mo</td>
</tr>
</tbody>
</table>

<sup>a</sup> Phase of therapy for the patient at the time the tumor specimen used to derive the cell line was obtained. The tumor stage at DX in all cases was stage IV.

<sup>b</sup> CTX, cyclophosphamide; DOX, doxorubicin; CDDP, cisplatin; CBDCa, carboplatin; VM-26, teniposide; ETOPO, etoposide; VCR, vincristine; L-PAM, melphalan; MTX, methotrexate; DTIC, dacarbazine; RAD, local irradiation; TBI, total body irradiation.

**Cytotoxicity Assay.** Melphalan, cisplatin, carboplatin, doxorubicin, and etoposide cytotoxicities were determined using the DIMSCAN assay system (17, 18). DIMSCAN employs digital imaging microscopy to quantify viable cells, which selectively accumulate FDA and thus are brightly fluorescent. DIMSCAN is capable of measuring cytotoxicity over a 4 log dynamic range by quantifying total fluorescence/well (which is proportional to viable, clonogenic cells) after eliminating background fluorescence with digital thresholding (17) and eosin-Y quenching (18). Cell lines were seeded in 150 µl complete medium at 15,000 cells/well into 96-well plates. After overnight incubation, various concentrations of chemotherapeutic drugs in 100 µl complete medium were added to each well. The concentration ranges used were: (a) cisplatin, etoposide, and doxorubicin, 0–10 µg/ml; and (b) carboplatin and melphalan, 0–12 µg/ml. Each condition was tested in replicates of 12. After incubation of cell lines with melphalan for 3 days, doxorubicin and etoposide for 4 days, and cisplatin or carboplatin for 7 days, 150 µl of medium was removed from each well. FDA in 50 µl of medium was then added (final FDA concentration was 8 µg/ml), plates were incubated for an additional 30 min at 37°C, and then 30 µl of 0.5% eosin Y (18) were added to each well. Total fluorescence was then measured using digital imaging microscopy and results were expressed as surviving fractions of treated cells compared with control cells. Dose-response curves plateau at high drug concentrations as a result of exceeding the 4 log dynamic range of the assay.

**RT-PCR.** To confirm a neuroblastoma origin for newly established cell lines (CHLA-15, CHLA-20, CHLA-42, CHLA-8, CHLA-79, and CHLA-134), TH expression (19) was determined by RT-PCR. To confirm a neuroblastoma origin for newly established cell lines (CHLA-15, CHLA-20, CHLA-42, CHLA-8, CHLA-79, and CHLA-90, and CHLA-134) were studied for binding of the neuroblastoma-associated monoclonal antibody HSAN1.2 and the anti-HLA class I antibody W6–32 (22). SMS-KAN, SMS-KANR, SMS-KCN, SMS-KCNR, SK-N-BE(1), SK-N-BE(2), SMS-SAN, SMS-LHN, and LA-N-6 have been characterized previously (22) for the expression of these antibodies. HSAN1.2, W6–32, or nonbinding IgG control antibodies (10 µg of antibody in 100 µl of PBS-GS = Dulbecco’s PBS without Ca<sup>2+</sup> or Mg<sup>2+</sup> (Irvine Scientific, Santa Ana, CA) supplemented with 5% goat serum (American Qualex, San Diego, CA) were added to 10<sup>5</sup> viable cells that were previously washed in PBS-GS. After incubation at 4°C for 30 min, cells were washed twice using PBS-GS and stained with 10 µl of fluorescein-labeled goat antimouse antiserum (FITC; American Qualex, San Diego, CA) diluted to 100 µl PBS-GS. Cells were incubated at 4°C in the dark for 30 min, washed with PBS-GS and then with PBS only, fixed in 1% paraformaldehyde (Mallinckrodt, Paris, KY) and analyzed on a FACStar Plus flow cytometer (Becton Dickinson, San Jose, CA), using Consort 30 software.

**Southern Blot Analysis.** MYCN amplification was studied both by Southern blot analysis and PCR. DNA was prepared from cultured cells as described in the manufacturer’s instructions (DNA extraction kit, Stratagene, La Jolla, CA). Ten µg of DNA samples were digested with restriction endonucleases, electrophoresed in a 0.8% agarose gel, and transferred to nylon membranes. Ten µg of human lymphocyte DNA was used as a standard for single-copy.

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Fig. 1. RT-PCR showing TH expression. A 299-bp fragment was amplified using primers as described in “Materials and Methods” and visualized by ethidium bromide staining. a. Lane 1, CHLA-15; Lane 2, CHLA-42; Lane 3, CHLA-20; Lane 4, CHLA-51; Lane 5, CHLA-8; Lane 6, CHLA-79; Lane 7, CHLA-90; Lane 8, CHLA-134; Lane 9, SK-N-BE(1); Lane 10, LA-N-6; Lane 11, CB-JFEN; Lane 12, CHP-100; Lane 13, N1001; and Lane 14, TC-71. SK-N-BE(1) and LA-N-6 were used as positive controls for TH expression. CB-JFEN is an esthesioneuroblastoma cell line; CHP-100 is a peripheral primitive neuroectodermal tumor cell line; and N1001 and TC-71 are Ewings sarcoma cell lines. b. β<sub>2</sub>-microglobulin was used to assess the quality of cDNA tested. The molecular weight marker is HaeIII-digested φX174.
intensity. Membranes were hybridized with 32P-labeled cDNA probe pNBl, a 1-kb BamHI-HindIII fragment from the 5'-end of the MYCN sequence (12). After hybridization at 65°C for 14–18 h, membranes were washed three times in 2X SSC at room temperature and additionally at 65°C for 1 h. Densitometric analysis of the filters was carried out using Phosphor Analyst software (Bio-Rad, Laboratories, Inc., Hercules, CA). Cell lines were defined to have MYCN amplification if the gene copy number of a cell line was ≥3 haploid genome by the Southern blot analysis (12). MYCN copy numbers for cell lines SMS-KAN, SMS-KCN, SK-N-BE(1), SMS-KANR, SMS-KCN, SK-N-BE(2), SMS-SAN, SMS-LHN, and LA-N-6, determined in this study by Southern blot analysis, differed from those studied by the dilution method (22). To correlate our data with data obtained by dilution method, we performed densitometric analysis of SMS-KCN at various dilution concentrations. The relationship between dilution coefficients and the MYCN copy numbers of SMS-KCN by Southern analysis was nonlinear, which explains the discrepancy between the data presented here and previous MYCN copy number determinations.

**PCR of MYCN**

Quantitative fluorescent PCR was also used to determine MYCN genomic amplification. Cell lines were considered to have MYCN amplification if the amount of MYCN DNA by PCR was >3 SDs above the mean of normal lymphocyte DNA (8017 ± 4664 from 10 separate lymphocyte samples). Therefore, cell lines with genomic MYCN >22.000 were considered to have MYCN amplification if the amount of MYCN DNA by PCR was >3 SDs above the MYCN genomic amplification. Cell lines were considered to have MYCN amplification. The primer sequence information (N-myc 7-1: 5'-GAT-... standard. An ABI Model 373A DNA Sequencer using GeneScan software (Applied Biosystems) was used for the precise quantitation and sizing of fluorescently-labeled PCR products. The ratio of the fluorescent peak areas of the 428-bp MYCN signal to the 396-bp signal was used as a measure of relative gene amplification, and the product of this ratio and of the input copy number of the 396-bp deletion fragment (25) is used to determine the absolute number of MYCN gene sequences in the sample.

For PCR, genomic DNA was isolated using TRIzol reagent (26). The cells were homogenized in the reagent; lysate was mixed with chloroform and centrifuged. DNA was precipitated from the interphase by the addition of ethanol and then was washed, denatured in 8 mM NaOH, and neutralized with 0.1 M HEPES solution. PCR was performed in a volume of 25 µl using 0.125 unit Taq polymerase (Perkin-Elmer, Roche Molecular Systems, Inc.) Amplification was performed in a Perkin-Elmer Cetus thermal cycler under the following conditions: (a) denaturation at 94°C for 1.5 min; (b) annealing at 55°C for 2 min; and (c) extension at 72°C for 3 min 30 cycles, followed by a final extension of 10 min at 72°C.

**Data Analysis.** Log(IC90) values (i.e., the drug concentrations that were cytotoxic for 90% of the cell populations) were calculated using the software "Dose-Effect Analysis with Microcomputers" (27). Fold-resistance for a given drug was defined as the ratio of the IC90 of a given cell line to the IC90 value of a sensitive cell line (SMS-SAN). A cell line with an IC90 value higher than the achievable clinical concentration (peak plasma level for agents administered as bolus injection or continuous steady-state drug concentration for continuous infusion agents) was considered resistant to that drug. The reported CSS for doxorubicin is 60 ng/ml (28) in patients treated with nonmyeloablative regimens. The CSS for etoposide ranges from 2 to 7 µg/ml (29-31) in patients treated with similar doses of etoposide as neuroblastoma patients on myeloablative regimens. The CSS for etoposide ranges from 2 to 7 µg/ml (29-31) in patients treated with similar doses of etoposide as neuroblastoma patients on myeloablative regimens (1). In this study, we used 5 µg/ml as a reference CSS for etoposide (29–31). In neuroblastoma patients treated with myeloablative doses, the CSS for carboplatin is 3 µg/ml and for cisplatin is 0.1 µg/ml, and the PPL for melphalan is 10 µg/ml.

Statistical significance of cross-resistance between cytotoxic agents was evaluated by the Pearson correlation analysis (32) employing the software SAS (SAS Institute, Inc., Cary, NC). Standardized residuals of each cell line, calculated as:

\[
\frac{\text{Log(IC90)}}{\text{Log(IC90)}} - \text{Av(Log(IC90))}_{\text{drug}}
\]

\[
\text{SD}
\]

Table 2 Concentrations of drugs that kill 90% of cells

<table>
<thead>
<tr>
<th>Phase of therapy* and cell lines</th>
<th>DOX&lt;sup&gt;a&lt;/sup&gt; (ng/ml)</th>
<th>ETO&lt;sup&gt;b&lt;/sup&gt; (ng/ml)</th>
<th>CDDP&lt;sup&gt;c&lt;/sup&gt; (µg/ml)</th>
<th>CBDCA (µg/ml)</th>
<th>L-PAM (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DX</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SMS-KAN</td>
<td>34.8</td>
<td>146</td>
<td>0.3</td>
<td>2.2</td>
<td>0.6</td>
</tr>
<tr>
<td>SMS-KCN</td>
<td>24.7</td>
<td>323</td>
<td>0.5</td>
<td>1.4</td>
<td>5.5</td>
</tr>
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<td>SK-N-BE(1)</td>
<td>&lt;0.1</td>
<td>158</td>
<td>&lt;0.1</td>
<td>0.2</td>
<td>0.8</td>
</tr>
<tr>
<td>CHIA-15</td>
<td>1.1</td>
<td>0.2</td>
<td>0.2</td>
<td>1.1</td>
<td>1.5</td>
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<tr>
<td>SMS-SAN</td>
<td>5.5</td>
<td>22.2</td>
<td>&lt;0.1</td>
<td>0.4</td>
<td>1.0</td>
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<td>CHLA-42</td>
<td>24.3</td>
<td>67.4</td>
<td>0.3</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>PD-Ind</td>
<td></td>
<td></td>
<td></td>
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<td>SMS-KANR</td>
<td>45.7</td>
<td>121</td>
<td>0.8</td>
<td>1.3</td>
<td>5.6</td>
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<td>SMS-KCNR</td>
<td>17.7</td>
<td>10.5</td>
<td>0.3</td>
<td>1.9</td>
<td>3.8</td>
</tr>
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<td>SK-N-BE(2)</td>
<td>92.3</td>
<td>1130</td>
<td>0.2</td>
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<td>CHLA-20</td>
<td>497.6</td>
<td>691</td>
<td>1.1</td>
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<td>CHAL-SHN</td>
<td>23.7</td>
<td>599.6</td>
<td>0.1</td>
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<td>LA-N-6</td>
<td>113.4</td>
<td>27.339</td>
<td>1.5</td>
<td>8.6</td>
<td>15.4</td>
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<tr>
<td>PD-BMT</td>
<td></td>
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<tr>
<td>CHLA-51</td>
<td>&lt;0.1</td>
<td>24.3</td>
<td>&lt;0.1</td>
<td>2.6</td>
<td>2.2</td>
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<tr>
<td>CHLA-8</td>
<td>8.2</td>
<td>41.8</td>
<td>0.7</td>
<td>2.3</td>
<td>0.3</td>
</tr>
<tr>
<td>CHLA-79</td>
<td>43.155</td>
<td>12.617</td>
<td>5.1</td>
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<td>CHLA-90</td>
<td>331.1</td>
<td>51.254</td>
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<td>375</td>
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<tr>
<td>CHLA-134</td>
<td>768.1</td>
<td>255.873</td>
<td>7.8</td>
<td>26.3</td>
<td>37.4</td>
</tr>
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</table>

* Phase of therapy for the patient when the tumor specimen used to derive the cell line was obtained.

<sup>a</sup> Results were obtained from data shown in Figs 2-4 and 6.

<sup>b</sup> DOX, doxorubicin; ETO, etoposide; CDDP, cisplatin; CBDCA, carboplatin; L-PAM, melphalan.
RESULTS

Cell Line Characterization. The cell lines used in this study, their in vivo exposure as tumors to drugs in patients, the sites from which the specimens were obtained, the stage of the disease, and the patient’s age at diagnosis are all shown in Table 1. MYCN amplification for the cell lines was determined by Southern blotting and confirmed by quantitative PCR (Table 4). The expression of TH for the newly established cell lines (CHLA-15, CHLA-42, CHLA-20, CHLA-8, CHLA-51, CHLA-79, CHLA-90, and CHLA-134), two previously characterized neuroblastoma cell lines [SK-N-BE(1) and LA-N-6], and nonneuroblastoma cell lines [esthesioneroblastoma (CB-JFEN), peripheral primitive neuroectodermal tumor (CHP-100), Ewing’s sarcoma (N1001 and TC-71)] is shown in Fig. 1. TH was expressed in all of the neuroblastoma cell lines but was not detected in the nonneuroblastoma cell lines.

The expression of cell surface antigens HSAN1.2 and W6–32 were determined by flow cytometry for CHLA-15, CHLA-42, CHLA-20, CHLA-8, CHLA-51, CHLA-79, CHLA-90, and CHLA-134. All of the cell lines showed positive binding for HSAN1.2 and negative or weak binding of W6–32, which is characteristic of neuroblastoma (data not shown).
**DRUG RESISTANCE IN NEUROBLASTOMA CELL LINES**

Carboplatin was 0.12 μg/ml and or cisplatin was 0.1 μg/ml. DX, cell lines established from patients at the time of diagnosis; PD-Ind, cell lines established from patients with progressive disease on conventional chemotherapy; PD-BMT, cell lines established from patients at the time of diagnosis; PD-lnii, cell lines established from patients with progressive disease on induction chemotherapy; PD-BMT, cell lines established from patients treated with etoposide. Etoposide, cisplatin, carboplatin, and melphalan. Results of cytotoxicity studies were expressed as IC₉₀ values for each drug tested (Table 2). These values were calculated from the dose-response curves shown in Figs. 2–4 and 6.

**Cytotoxicity Studies.** We determined the sensitivities of the cell line panel to drugs commonly used for neuroblastoma: doxorubicin, etoposide, cisplatin, carboptatin, and melphalan. Results of cytotoxicity assays were expressed as IC₉₀ values for each drug tested (Table 2). These values were calculated from the dose-response curves shown in Figs. 2–4 and 6.

Dose-response curves for the panel of neuroblastoma cell lines to doxorubicin are shown in Fig. 2. All of cell lines established at progressive disease during induction chemotherapy (PD-Ind) and cell lines established at relapse after myeloablative therapy (PD-BMT) were derived from patients treated with doxorubicin (Table 1). As shown in Table 2, the IC₉₀ for doxorubicin generally increased with the intensity of therapy: DX cell lines showed an IC₉₀ of <0.1–35 ng/ml, PD-Ind cell lines 17–498 ng/ml, and PD-BMT cell lines <0.1–43,155 ng/ml. The doxorubicin IC₉₀ was higher than the clinically achievable steady state drug concentration (CSS) in patients treated with nonmyeloablative doses for 0 of 6 DX cell lines, 3 of 6 PD-Ind cell lines, and 3 of 5 PD-BMT cell lines. CHLA-20 had the highest doxorubicin IC₉₀ value within the PD-Ind group, which was eight times higher than the CSS. For the doxorubicin-resistant PD-BMT cell lines, the IC₉₀ values were 719 (CHLA-79), 6 (CHLA-90), and 13 (CHLA-134) times higher than the reported CSS.

Dose-response curves of cell lines to etoposide are shown in Fig. 3. Etoposide or another epipodophyllotoxin, teniposide, was used to treat patients from whom two PD-Ind and all of the PD-BMT cell lines were derived (Table 1). Etoposide IC₉₀ values ranged from 0.2 to 323 ng/ml for DX cell lines, from 11 to 27,339 ng/ml for PD-Ind cell lines, and from 24 to 255,873 ng/ml for PD-BMT cell lines. The IC₉₀ for etoposide was higher than the CSS for 0 of 6 DX, 1 of 6 PD-Ind, and 3 of 5 PD-BMT cell lines. Generally, resistance to etoposide was found in PD-Ind (LA-N-6) and PD-BMT (CHLA-79, CHLA-90, and CHLA-134) cell lines derived from patients treated with etoposide. However, two PD-BMT cell lines, CHLA-8 and CHLA-51, which were derived from etoposide-treated patients, were not resistant. The etoposide IC₉₀ for the resistant PD-Ind cell line (LA-N-6) was six times higher than the reported CSS. Etoposide resistant PD-BMT cell lines showed IC₉₀ values 3 (CHLA-79), 10 (CHLA-90), and 51 (CHLA-134) times higher than the CSS.

Dose-response curves for carboplatin and cisplatin are shown in Fig. 4. The dose-response to cisplatin is steeper than for carboplatin. This observation might lead to the impression that cisplatin is more cytotoxic than carboplatin. However, a comparison of surviving fractions at the clinically achievable CSS (3 μg/ml for carboplatin and 0.1 μg/ml for cisplatin in patients treated with myeloablative doses) showed that, for many cell lines, carboplatin was more cytotoxic than cisplatin at the levels achievable in patients (Fig. 5). The IC₉₀ for carboplatin was higher than the achievable CSS for 0 of 6 DX, 2 of 6 PD-Ind, and 3 of 5 PD-BMT cell lines. By contrast, cisplatin IC₉₀ values found in PD-Ind (LA-N-6) and PD-BMT (CHLA-79, CHLA-90, and CHLA-134) cell lines were higher than the CSS. The carboplatin IC₉₀ for DX cell lines ranged from 0.2 to 2.2 μg/ml, for PD-Ind cell lines from 1.3 to 8.6 μg/ml, and for PD-BMT cell lines from 2.3 to 26.3 μg/ml. The cisplatin IC₉₀ for DX cell lines ranged from 0.3 to 0.5 μg/ml, for PD-Ind cell lines from 0.2 to 1.5 μg/ml, and for PD-BMT cell lines from <0.1 to 7.8 μg/ml. The IC₉₀ values of carboplatin-resistant

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**Fig. 4.** Dose-response curves to carboplatin (•) and cisplatin (○) obtained by DIMSCAN analysis of neuroblastoma cell lines are shown. The concentration range tested for carboplatin was 0–12 μg/ml and for cisplatin was 0–10 μg/ml. DX, cell lines established from patients at the time of diagnosis; PD-Ind, cell lines established from patients with progressive disease on conventional chemotherapy; PD-BMT, cell lines derived from patients who relapsed after myeloablative chemoradiotherapy followed by BMT.

We confirmed by DNA fingerprinting that PD-Allo-BMT cell lines, CHLA-8 and CHLA-79, matched the tumor specimens from the patients who relapsed after myeloablative chemoradiation followed by BMT. Cell lines derived from patients treated with etoposide, cisplatin, carboplatin, and melphalan. Results of cytotoxicity studies were expressed as IC₉₀ values for each drug tested (Table 2). These values were calculated from the dose-response curves shown in Figs. 2–4 and 6.

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**Fig. 5.** Surviving fractions at clinically achievable drug concentrations: carboplatin, 3 μg/ml (●); cisplatin, 0.1 μg/ml (○). These results were derived from DIMSCAN analysis of neuroblastoma cell lines treated over a wide concentration range of each drug. DX, cell lines established from patients at diagnosis; PD-Ind, cell lines established from patients at diagnosis; PD-BMT, cell lines established from patients with progressive disease during induction chemotherapy; PD-BMT, cell lines established from patients after myeloablative chemoradiotherapy and BMT.
PD-Ind cell lines were >1 (CHLA-20) and three (LA-N-6) times higher than the CSS, and of carboplatin-resistant PD-BMT cell lines were >one (CHLA-79), five (CHLA-90), and nine (CHLA-134) times higher than the CSS. The IC₉₀ values of cisplatin-resistant DX cell lines were three (SMS-KAN, CHLA-42), five (SMS-KCN), and two (CHLA-15) times higher than the cisplatin CSS, and cisplatin-resistant PD-Ind cell lines had IC₉₀ values 8 (SMS-KANR), 3 (SMS-KCNR), 2 (SK-N-BE(2)), 11 (CHLA-20), and 15 (LA-N-6) times higher than CSS. Cisplatin-resistant PD-BMT cell lines had IC₉₀ values 7 (CHLA-8), 51 (CHLA-79), 25 (CHLA-90), and 78 (CHLA-134) times higher than the achievable CSS.

The cytotoxicity of melphalan over a range of concentrations for the cell line panel is shown in Fig. 6. Melphalan IC₉₀ values were higher than PPL achieved by myeloablative doses of the drug for 0 of 6 DX, 2 of 6 PD-Ind, and 2 of 5 PD-BMT cell lines. Melphalan IC₉₀ values for DX cell lines ranged from 0.5 to 6 µg/ml for PD-Ind cell lines 0.5 to 24 µg/ml, and for PD-BMT cell lines 0.3 to 375 µg/ml. The melphalan IC₉₀ for the resistant PD-Ind cell lines (SK-N-BE(2) and LA-N-6) was two times higher than PPL. Melphalan-resistant PD-BMT cell lines showed IC₉₀ values 38 (CHLA-90) and 4 (CHLA-134) times higher than the PPL.

The degree of drug resistance/sensitivity for the panel of neuroblastoma cell lines to cisplatin, carboplatin, melphalan, doxorubicin, and etoposide is summarized in Fig. 7. Fold-resistance calculated for each cell line was used to compare resistance/sensitivity of the cell lines for each cytotoxic agent. Most cell lines established from patients before treatment were sensitive to most drugs, whereas resistance was increased in cell lines established after induction chemotherapy, and it was highest in those cell lines established from patients after intensive chemoradiotherapy.

Drug Resistance Phenotype in DX and PD-Ind Paired Cell Lines. Within the panel of 17 neuroblastoma cell lines studied, there were pairs of cell lines derived from the same patients (four different patients) at diagnosis and then after progression during induction therapy: (a) SMS-KAN + SMS-KANR; (b) SMS-KCN + SMS-KCNR; (c) SK-N-BE(1) + SK-N-BE(2); and (d) CHLA-15 + CHLA-20. PD-Ind cell lines SMS-KANR and SMS-KCNR were derived from patients treated with cyclophosphamide and doxorubicin (Table 1). SMS-KANR and SMS-KCNR did not show increased drug resistance relative to the DX cell lines derived from the same patients (Table 2). Thus, in 2 of 4 pairs, there was no increase in drug resistance in PD-Ind cell lines compared with DX cell lines.

SK-N-BE(2) was derived from a patient after treatment with cyclophosphamide, doxorubicin, and vincristine (Table 1). SK-N-BE(2) showed increased drug resistance relative to SK-N-BE(1), a DX cell line from the same patient (Table 2). For example, the IC₉₀ values for SK-N-BE(2) were greater than IC₉₀ values for SK-N-BE(1) by 9230 times for doxorubicin, 7 times for etoposide, 9 times for cisplatin, 13 times for carboplatin, and 30 times for melphalan.

CHLA-20 was derived from a patient after treatment with cyclophosphamide, doxorubicin, cisplatin, and teniposide (Table 1), and IC₉₀ values for this cell line were higher than clinically achievable levels for doxorubicin, cisplatin, and carboplatin (Table 2). CHLA-20 showed increased resistance to doxorubicin, cisplatin, and carboplatin when compared with CHLA-15, a DX cell line from the same patient. IC₉₀ values for CHLA-20 were greater than those for CHLA-15 by 436 times for doxorubicin, 3839 times for etoposide, 4.9 times for cisplatin, and 3.5 times for carboplatin.

Cross-Resistance Patterns. Statistical significance of cross-resistance was evaluated for neuroblastoma cell lines by the Pearson correlation test (Table 3). Significant cross-resistance was found between: (a) cisplatin and doxorubicin (r = 0.88; P = 0.0001); (b) cisplatin and carboplatin (r = 0.71; P = 0.001); (c) carboplatin and etoposide (r = 0.69; P = 0.002); (d) carboplatin and melphalan (r = 0.72; P = 0.002); (e) etoposide and cisplatin (r = 0.66; P = 0.004); (f) etoposide and doxorubicin (r = 0.61; P = 0.01); and (g) melphalan and etoposide (r = 0.65; P = 0.04).

Drug Resistance Increased with Therapy. The IC₉₀ values of the cell lines significantly increased with therapy (from DX to PD-Ind and PD-BMT): (a) doxorubicin (P = 0.04); (b) etoposide (P = 0.03); (c) cisplatin (P = 0.03); (d) carboplatin (P = 0.002); and (e) melphalan (P = 0.04; one-sided two-sample t test).

Although most cell lines were tested at similar passage number (mid 20s), the range was from passage 3 to 40. Using a regression analysis of sequential sums of squares with passage number, we found that the increase in resistance of cell lines established after therapy was significant if we controlled for cell line passage number.

MYCN Amplification in Cell Lines. In 4 of 6 DX, 3 of 6 PD-Ind, and 2 of 5 PD-BMT cell lines, MYCN genomic amplification was detected (Table 4). The two-tail Fisher's exact test did not show correlation between MYCN amplification and resistance to doxorubicin (P = 0.33), etoposide (P = 0.29), carboplatin (P = 0.13), cisplatin (P = 1), or melphalan (P = 1).
Neuroblastoma initially responds to chemotherapy and then recurs as chemotherapy-insensitive (4, 35). Development of chemotherapy-refractory disease in patients whose tumors showed an initial response to chemotherapy suggests that in vivo selection of drug-resistant tumor cells occurred during therapy. Drug resistance in cancer has been largely investigated by in vitro selection of resistant cell lines with continuous or intermittent exposure of cells to the chemotherapeutic agents (5, 36) or by association of drug-resistance genes with clinical outcome of patients (5-7, 37). In the present work, we investigated whether neuroblastomas treated in patients and then established as cell lines manifest a sustained drug-resistant phenotype and whether drug resistance correlates with the treatment that patients had received. Neuroblastoma cell lines used in this study were derived from patients at diagnosis before treatment or after treatment with chemotherapy and whether drug resistance correlates with the treatment that patients had received. Neuroblastoma cell lines were derived from patients at diagnosis before treatment or after treatment with chemotherapy exposure were sensitive to most of the cytotoxic agents tested in vitro. Resistance was defined for five cytotoxic agents commonly used for neuroblastoma by comparing the cell-kill ability of each drug with the clinically achievable concentrations of the agent.

Cell lines established from patients at the time of diagnosis before chemotherapy exposure were sensitive to most of the cytotoxic agents tested in this study (cisplatin being the only exception). Increased resistance to one or more drugs was detected in cell lines isolated at the time of disease progression in patients undergoing nonmyeloablative induction chemotherapy. The highest levels of resistance were demonstrated in cell lines obtained from patients treated with intensive myeloablative therapy and BMT. The IC\textsubscript{50} values of most cytotoxic agents for three of five PD-BMT cell lines were many-fold higher than clinically achievable drug concentrations: 3–51 times for etoposide, 6–719 times for doxorubicin, 25–78 times for cisplatin, >1–9 times for carboplatin, and 0–38 times for melphalan. In the current Children’s Cancer Group (CCG) consolidation protocols for neuroblastoma, doxorubicin is omitted and cisplatin is replaced by carboplatin. The high resistance to etoposide and doxorubicin in cell lines exposed to these drugs in patients suggests that etoposide and doxorubicin may mostly contribute to drug-related toxicities without effectively contributing to tumor cell-kill in many patients when used late in the course of therapy. Thus, consideration may be given to substituting novel non-cross-resistant agents for etoposide and doxorubicin in the consolidation phase of treatment.

Drug resistance patterns expressed by neuroblastoma cell lines generally correlated with the treatment intensities and the drugs that the patients were exposed to. The PD-Ind cell lines—SMS-KANR, SMS-KCNR, and SMS-LHN—were established from patients treated with only cyclophosphamide and doxorubicin (15, 16). SMS-KANR and SMS-KCNR cell lines did not show increased drug resistance relative to DX cell lines from the same patients. Higher IC\textsubscript{50} values and various levels of resistance to all of the drugs were seen in cell lines SK-N-BE(2), CHLA-20, and LA-N-6. SK-N-BE(2) was derived from a patient treated with vincristine, cyclophosphamide, doxorubicin, and radiation (14), and CHLA-20 was derived from a patient treated with cisplatin, teniposide, cyclophosphamide, and doxorubicin. A cell line with the highest drug resistant phenotype among PD-Ind cell lines (LA-N-6) was isolated from a patient who received multiple courses of chemotherapy containing cyclophosphamide, doxorubicin, teniposide, etoposide, vincristine, and dacarbazine due to progressive disease (16). Moreover, higher drug resistance was seen in cell lines derived from patients treated with myeloablative chemotherapy and BMT. Thus, expression of a drug resistant phenotype in neuroblastoma cell lines appears to increase with the intensity of chemotherapy given to the patients.

Table 3  Cross-resistance among drugs expressed as r values from the pearson correlation test

<table>
<thead>
<tr>
<th>Drug</th>
<th>CBDDCA</th>
<th>CDDP</th>
<th>L-PAM</th>
<th>DOX</th>
<th>ETOP</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBDDCA</td>
<td>1</td>
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</table>
| CDDP       | 0.71 (0.001)
| L-PAM      | 0.59 (0.002)
| DOX        | 0.55 (0.002)
| ETOP       | 0.69 (0.001)

* Values of r > 0.6 were considered significant cross-resistance.
Resistance to etoposide was observed only in cell lines derived from patients exposed to etoposide and/or teniposide (LA-N-6, CHLA-79, CHLA-90, and CHLA-134). Resistance was also seen in some cell lines with platinum compounds and melphalan. Carboplatin-resistant cell lines showed IC₅₀ values 1.1–9 times higher than clinically obtainable steady state concentration (CSS), whereas cisplatin IC₅₀ values were 2–78 times higher than the CSS. Thus, at clinically achievable levels, resistance to carboplatin is lower than resistance to cisplatin in vitro. In the panel of seventeen cell lines, only four demonstrated resistance to melphalan: SK-N-BE(2), LA-N-6, CHLA-90, and CHLA-134. LA-N-6 was derived from a tumor that had been exposed to a variety of alkylating agents, whereas SK-N-BE(2) came from a tumor exposed to cyclophosphamide and radiation. CHLA-90 and CHLA-134 cell lines were obtained from patients who received melphalan as a part of a myeloablative consolidation regimen before BMT.

Our experience with these cell lines showed that drug resistance is maintained in resistant cell lines in higher passages (over 40) and that sensitive cell lines remain sensitive from lower to higher passages and the increase in drug resistance seen from diagnosis to posttherapy cell lines was independent of cell line passage number. Passage number did not account for the difference in IC₅₀ values seen in the cell line panel from DX to PD-Ind and PD-BMT groups.

This is the first study characterizing cell lines obtained from patients who underwent intensive myeloablative chemotherapy and BMT. Very high drug resistance was seen in both PD-Allo-BMT and PD-Auto-BMT cell lines. One of two PD-BMT cell lines established after allogeneic BMT and two of three cell lines established after autologous BMT showed high resistance to all of the five cytotoxic agents tested in this study. The sustained high levels of drug resistance in most of these PD-BMT cell lines points to intrinsic alterations in tumor cells as a mechanism for PD-BMT relapse and suggests that the use of drug-resistance modulating agents and/or drugs that are not cross-resistant with agents used for induction chemotherapy will improve myeloablative consolidation regimens.

Aggressive behavior of neuroblastoma is observed in patients with MYCN amplification (13), and may also be an indicator of poor response to chemotherapy, thus suggesting some role for MYCN in the acquisition of chemotherapy-refractory disease (38). In our study, we could not establish an association of MYCN amplification with drug resistance by the two-tail Fisher’s exact test. Some cell lines with MYCN amplification showed drug resistance, and some MYCN-nonamplified cell lines were sensitive to drugs. Nevertheless, there were highly resistant MYCN-nonamplified lines and chemotherapy-sensitive MYCN-amplified cell lines. Thus, MYCN amplification does not appear to confer drug resistance by itself.

A number of studies have used cell lines of various tumor types that were selected in vitro for drug resistance and then maintained in the absence of the selecting agent to monitor reversal of the drug-resistant phenotype and associated genetic changes. The reversal of drug resistance has been noted whether or not concomitant genetic alterations have occurred (39, 40). By contrast, our cell lines, which were not selected for resistance in vitro, were maintained in drug-free medium, and they showed sustained drug resistance to various classes of cytotoxic agents that correlated with the therapies given to the patients. Similar to our findings, increased resistance to cisplatin and doxorubicin in a posttherapy neuroblastoma cell line relative to a pretherapy cell line from the same patient had been reported earlier (41). The cell line panel described in this report should be ideal for studying the molecular changes that confer drug resistance in neuroblastoma in vivo.

Cross-resistance patterns between various classes of drugs exhibited by the cell line panel suggest that multiple drug resistance mechanisms are involved. Determination of the molecular aspects of drug resistance observed in our model system is beyond the scope of this report because of the complexity and multiplicity of these mechanisms. It has previously been shown that mdr-1 (5, 6, 42, 43) and MRP (7, 44) play a role in resistance to natural product drugs. Other mechanisms associated with resistance include expression of lung resistance-related gene (LRP; Ref. 45), decreased levels (46) or mutations (47) of topoisomerase II, and increased expression of Bcl-2 or Bcl-xL (48-50). Glutathione and related enzymes (39, 41, 51), in increased DNA repair (52, 53), and reduced drug accumulation (non-mdr-mediated; Ref. 54), have all been associated with resistance to platinum compounds and classical alkylating agents in vitro. Studies of these molecular mechanisms in our cell line panel are in progress.

Finally, the cell lines described here provide a unique panel for preclinical studies aimed at identifying non-cross-resistant agents for new clinical trials. They should be helpful in designing clinical strategies to overcome drug resistance developed during the course of treatment by identifying agents active against drug-resistant tumor cells.

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Drug Resistance Patterns of Human Neuroblastoma Cell Lines Derived from Patients at Different Phases of Therapy

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