Expression of N-myc, c-myc, and MDR-1 Proteins in Newly Established Neuroblastoma Cell Lines: A Study by Immunofluorescence Staining and Flow Cytometry

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ABSTRACT

A methodology for rapid isolation of neuroblastoma cells from marrow with metastatic neuroblastoma cells was developed using a cocktail of five antibodies and magnetic microspheres coated with secondary antibodies. Cells bound to microspheres were released by brief exposure to chymopapain, followed by repeated culture of released cells in serum-supplemented Dulbecco’s modified Eagle’s medium and selection for adherent cells. Using this methodology, over 35 primary cell lines were obtained free of contaminating normal cells. Detailed analyses of over 14 cell lines revealed gross differences in cell phenotype, size, morphology development of neurite processes, and doubling time (40 to 80 h). All cell lines expressed the N, 145,000 neurofilament, and a few expressed the M, 200,000 neurofilament, with very little or no expression of the M, 68,000 neurofilament. Eighty % of all cell lines had near-diploid DNA content. High expression of the MDR-1 protein was detected in six of the 22 cell lines tested.

Great heterogeneity was observed in the expression of N-myc oncoprotein, with ten of 13 patients overexpressing the protein. c-myc oncoprotein was also expressed in all cell lines; however, the level of expression was 4- to 10-fold lower than the N-myc oncoprotein. Localization studies of c-myc and N-myc oncoproteins on the level of light microscopy and electron microscopy revealed exclusive nuclear localization of c-myc, whereas N-myc was localized to the nucleus and to the cytoplasm.

INTRODUCTION

NB1 is a cancer of the sympathetic nervous system, affecting mainly children and young adults. It is the commonest solid tumor in children, and over 400 new cases are detected each year in the United States. The most important prognostic factors are age, clinicopathological stage, expression of N-myc oncogene, and DNA ploidy. Patients with metastasis to the bone marrow (Stage 4) with a high level of N-myc expression have, in general, a bad prognosis. However, 60% of the cases with poor prognosis do not have elevated N-myc gene copies or N-myc RNA transcripts (1-6). In recent publications evidence was presented that overexpression of the N-myc protein is not always correlated with gene copies or RNA transcripts in patient biopsies (7-10). Coexpression of L-myc, c-myc, and N-myc mRNA transcripts in neuroblastoma cells was reported recently; however, correlation between myc gene expression and progression of disease was found only when the N-myc gene was amplified (7, 11-13).

Received 8/19/91; accepted 2/26/92.

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1 This work was supported partly by a grant from “STOP! Children’s Cancer, Inc.” and partly by the Division of Sponsored Research of the University of Florida (Y. G.).

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3 The abbreviations used are: NB, neuroblastoma; MDR, multiple drug resistance; ABM, autologous bone marrow transplantation; PBS, phosphate-buffered saline; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; IgG, immunoglobulin G; IgM, immunoglobulin M; NF, neurofilament; FITC, fluorescein isothiocyanate; PE, phycoerythrin; mpc, mean peak channel; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; ABC, avidin-biotin complex; ELISA, enzyme-linked immunosorbent assay; POG, Pediatric Oncology Group.

Diploid content of DNA and deletion of the short arm of chromosome 1 have also been found to correlate with advanced disease (4, 7, 14-17). Elevation of N-myc correlated slightly better with disease progression than did DNA ploidy of NB cells (7, 18-20).

Expression of the MDR-1 gene in various cancer cells has recently been found to correlate with clinical response to therapy and has been used to predict relapse in over 50% of patients (21). Similar correlation was noted in NB patients, studied recently. In general, positive correlation was found between disease progression and expression of the MDR-1 gene in 25 to 35% of the patients (22).

In this paper we describe a new approach to isolating and establishing NB cell lines from bone marrow of patients undergoing immunomagnetic removal of tumor cells for ABMT. As a referral center for bone marrow purging, we have access to over 60 marrows a year. Cell lines were characterized in terms of cell phenotype and morphology, expression of neurofilament proteins, expression of the oncoproteins N-myc and c-myc, and expression of the MDR-1 protein. Expression of N-myc, MDR-1 proteins, and DNA ploidy is correlated with relapse following ABMT.

MATERIALS AND METHODS

Release of NB Tumor Cells from Microspheres

Tumor cells were released by incubating the cell-microsphere complexes (bone marrow “purgate”) with 10 units/ml of chymopapain (Chymodiactin; Boots Pharmaceuticals, Lincolnshire, IL) at 37°C in PBS for 20 to 30 min. The released cells were then separated from the microspheres by adherence to a samarium cobalt magnet, washed 3 times with medium, and resuspended in DMEM containing 40% FCS. Up to 35 million cells can be recovered from the microspheres derived from 300 to 400 ml of processed marrow.

Cell Culture

All primary cultures are carried out in DMEM + 40% FCS (Gibco, Grand Island, NY). After reaching confluence, adherent cells were transferred to T-75 flasks and diluted 1:3 with fresh medium, leaving 10 to 20% conditioned medium every 6 to 10 days depending on the rate of growth of the cell line. Nontumorous cells (mostly fibroblasts) disappear after the first few weeks. Cells were dislodged for cell transfer by brief exposure to trypsin-EDTA (Gibco). Established NB cell lines (CHP 120, 50 copies of the N-myc gene) (23, 24) and SK-N-SH (American Type Culture Collection, HTB11, one copy of the N-myc gene) (16) were cultured in RPMI medium containing 10% FCS (Gibco) in T-75 flasks. Confluent cultures were transferred following dislodging of cells with trypsin-EDTA. The population doubling time was derived from multiple cultures seeded at 0.5 x 10^6 cells/flask in 5 ml of medium, harvested, and counted every 24 h. The doubling time was calculated from the linear portion of the growth curve on a semilog plot.

Antibodies

Anti c-myc antibody (IgG1) was obtained from hybridoma tissue culture of a National Cancer Institute clone specific for amino acid...
peptide residue 171-188 of the c-myc oncoprotein.

Anti N-myc antibody (IgM) was generated in our laboratory by immunization of BALB/c mice with N-myc-specific amino acid peptide residue 336-348. Specificity of antibody was determined by Western blot analysis and by specific staining of established neuroblastoma cell lines with a known amplification of the N-myc oncogene and with leukemic lines which express very low levels of N-myc (see “Results”).

Anti-neurofilament antibodies (NF-L, NF-M, NF-H, all IgG) were purchased from Oncogene Sciences (Manhasset, NY).

Anti-NB antibodies U131A and H11 (anti-N-CAM), antibody 127.1 (anti-L-1), anti-Thy-1, and M340, all specific for neuronal cells, were obtained from Dr. John Kem shead, Bristol, United Kingdom.

Anti-MDR-1 antibody (Hyb 241, IgG1) was a gift from Dr. Lena Grauer, Hybritech, CA.

Chymopapain

Chymopapain was a gift from Dr. Steven Schofield, Boots Pharmaceuticals, Lincolnshire, IL.

Immunofluorescence Staining

Staining for N-myc and c-myc Oncoproteins. Staining was carried out as previously described for leukemic cells (25, 26) with slight modifications. Cells were deslodged from flasks by shaking (1 to 5 min) treatment with 5 ml of trypsin-EDTA (Gibco), washing twice with PBS containing 4% FCS, and then fixing them with 1 ml of 1% paraformaldehyde for 20 min. Cells were then washed and permeated with 0.1% Triton X-100 in PBS-4% FCS for 20 min. Staining was performed on 0.1 to 0.5 × 10^6 cells/tube with 100 μl of hybridoma supernatants. Following 30-min incubation on ice, tubes were washed once with the same buffer, and secondary antibody [sheep anti-mouse IgG (Fc)-specific FITC for c-myc or sheep anti-mouse IgM-PE specific for N-myc] was added for 30 min on ice. Unbound secondary antibody was removed by washing as before. Both secondary antibodies were affinity-purified Fab fragments, absorbed on animal sera (Jackson Immunochemicals, Mt. View, CA). Mouse IgG and mouse IgM controls at 5-fold excess were used for each staining combination to determine nonspecific binding. All cell staining was done at antibody-saturating concentrations. In staining of newly established NB cell lines, the CHP-126 cell line was always added as a positive control. The mpc of CHP-126 varied ±8 units between different experiments.

Staining for NB Surface Antigens. Staining was performed on intact cells, removed from flasks by scraping the cells with a rubber policeman. Primary antibodies were added at a concentration of 2 μg/10^6 cells/100 μl of PBS ± 4% FCS for 30 min at 4°C. Unbound antibody was removed by centrifugation, 5 μl of secondary antibody (FITC-sheep anti-mouse IgG) were added, and the cells were incubated for 30 min at 4°C. Unbound secondary antibody was removed by centrifugation, and stained cells were analyzed by flow cytometry (see below). Mouse IgG1 served as control for nonspecific binding.

Staining for MDR-1 Protein. Staining was performed essentially as described for NB surface antigens using 5 μg of Hyb 241 anti-MDR-1 antibody as primary antibody. Specific binding was calculated as the percentage of positive cells above the isotypic control (~2%). CEM and CEM/VLB-100 (provided by Dr. Beck, St. Jude Children’s Hospital, Memphis, TN) served as negative and positive control cell lines, respectively (27). Cell lines with over 10% positive cells at a mpc of >15.0 were considered positive for MDR-1.

Staining of NB Cells Fixed in Petri Dishes. Cells growing in a Petri dish were fixed by brief exposure to 1% paraformaldehyde followed by permeation with 0.1% Triton X-100. Primary antibodies (antineurofilaments; anti-c-myc/N-myc) were added as with cells in suspension. Micrographs were taken as described below for staining with vital signs. A green filter was used for FITC, and a red filter was used for phycocyanin-stained cells.

Staining with Vital Stains. Hoechst and fluorescein diacetate were used to stain nuclei and cytoplasm, respectively, of living NB cells. Cells growing in Petri dishes were stained by brief exposure to the dye and subsequently photographed for green fluorescence (FITC filter) and blue fluorescence (UV filter) using a Nikon fluorescence microscope equipped with an FX-35 camera and an AF-X II automatic exposure device.

Staining with Immunogold-labeled Antibodies and Electron Microscopy

Postembedding Labeling. Cells were harvested by centrifugation and resuspended in a mixture of 0.1% glutaraldehyde, 4% freshly prepared paraformaldehyde, and 50 mM lysine (free base) in PBS and placed in an ice bath for 30 min. The cells were washed for 3 cycles of 15 min in PBS on ice, dehydrated, and embedded in LR Gold according to the supplier's instructions (Polysciences, Inc., Warrenton, PA). Thin sections were cut and mounted on Pioiloform-coated nickel grids and incubated overnight, at 4°C, with hybridoma supernatant diluted 1:5 in 0.5 M NaCl/0.2 M Tris-HCl (pH 7.2);0.1% TWEEN 20. Grids were washed twice in this complex and once with PBS prior to incubation with 15 nm of gold-anti-mouse IgG (E-Y Laboratories, San Mateo, CA) for 1 h at room temperature. The grids were washed twice in PBS and once in distilled water. They were observed unstained or lightly stained with uranyl acetate and lead citrate.

Staining for DNA

Staining for DNA was done as described before (28) with propidium iodide. The diploid mean peak channel of 2C was adjusted by running normal resting lymphocytes, where 98% of cells are in G1. Results are presented as the DNA index relative to normal diploid lymphocytes (index of 1 in diploid tumors) and up to tetraploid tumor cells with a DNA index of 2.0.

Flow Cytometry

Coulter EPICS Profile II was used to analyze stained cells. Stained samples with green fluorescence (c-myc) or for red fluorescence (N-myc) were run against their isotypic control at 800 V. Log fluorescence was used, and 5000 cells were analyzed. For two-color immunofluorescence 10% compensation of red over green was used, and 4% compensation was used to correct spill of green to red fluorescence. All samples were run within the same day. The average background staining of the IgG isotypic control was less than 1% with an mpc of 1 to 2. The average background staining for IgM isotypic control was 1 to 2% at an mpc of 2.5. Analysis of two-color staining (green + red) was obtained by using an adequate built-in program.

Cells stained for MDR-1 and NB surface markers were analyzed similarly using 800 V on the green fluorescence PMT. Five thousand cells were analyzed.

Western Blot Analysis

CHP-126 cells (5 × 10^6) were washed with PBS (3 times), and the cell pellet was digested with 10 mM Tris-HCl buffer (pH 7.4) containing 5 mM MgCl₂, 0.5% SDS, 0.5% deoxycholate, 0.5% Nonidet P-40, 10 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. Protein content was determined by the BCA (Pierce, Rockford, IL) method, and 50 μg of protein were run on a 9% SDS-PAGE according to the method of Laemmli (29). Proteins were transferred to Immobilon-P transfer membrane (Millipore, Bedford, MA) using Biorad Transblot-SD semi-dry electrophoretic transfer cell (Richmond, CA) at 25 V for 45 min, in Bjerrum and Schafer-Nielsen transfer buffer (48 mM Tris:39 mM glycine:1.3 mM SDS:20% methanol). Transfer paper was then blocked 1 h with 4% BSA + 0.5% Tween 20 in 50 mM Tris-HCl buffer (pH 8.0). Hybridioma culture supernatant (undiluted) from clone 5C6 or IgM control was then added overnight at 4°C with gentle rocking.

Unbound antibody was then washed with 3 changes of PBS containing 0.5% BSA and 0.5% Tween 20. Biotin-goat anti-mouse IgG was then added for 2 h followed by avidin:peroxidase (ABC kit; Vector Laboratories, Inc., Burlingame, CA), with 4-chloro-1-naphthol reagent as a substrate. Biotinylated standard protein markers were included in each run (BSA, M, 68,000; carbonic anhydrase, M, 30,000). Nontransferred gels were stained with Coomassie brilliant blue and destained with 10% acetic acid.
RESULTS

Establishment of NB Cell Lines from Bone Marrow Metastases. Table 1 is a partial list of 35 NB cell lines newly established from purged marrow of Stage 4 NB patients in complete clinical remission. Neuroblastoma cells were removed from patients' marrow by the immunomagnetic method, using a cocktail of 5 anti-NB antibodies and magnetic microspheres coated with anti-mouse IgG (4.5 µm; Dynal, Great Neck, NY) as described before (30, 31). Purged marrow as reinfused to the patients, whereas NB cells bound to microspheres and trapped on a samarium-cobalt magnet were collected, washed with PBS, and released from microspheres by chymopapain treatment of microspheres. Usually over 90% of the cells were viable. Cells appeared to be large and homogeneous in size, with characteristic granularity. The cell phenotype was determined following 1 to 7 wk in culture, depending on the number of cells recovered. Detailed analysis of 18 different bone marrow purges revealed an average of 12.1 x 10^6 NB tumor cells (range, 1.7 x 10^6 to 35 x 10^6 cells), where the average number of tumor cells, by immunofluorescence staining for 5 anti-NB antibodies, was 0.11% (range, 0.0 to 0.5%) with 10 of 18 marrows with undetectable tumor cells before the purging procedure.

Table 1 summarizes the cell phenotype and doubling time of cells derived from 9 NB patients. Great heterogeneity in the expression of the different antigens was noted in cells derived from different patients. The distribution of two N-CAM adhesion antigens (5.1 H11 and UJ13A) was fairly similar and different from the antigens detected by antibody M-340, UJ 127.11, or UJ 181.4. In contrast, the Thy-1 antigen is highly expressed in all of the cell lines tested. The use of a cocktail of 5 different antibodies resulted in binding of over 99% of the cells. The doubling time varies between 40 h (S. F.) and 80 h (B. D.). The distribution of cell surface antigens and the doubling time of an established cell line (CHP-126) are also shown in Table 1.

Generation of Anti-N-myc Antibody and Detection of the Expression of N-myc and c-myc Oncoproteins. Fig. 1 depicts the sequence of the 13-amino acid peptide in exon 3 of the N-myc gene (amino acid 336–346) (32, 33). The peptide was synthesized by Pep Tech, Inc. (Alachua, FL), using a Biosearch 9500 synthesizer. The peptide was then analyzed for its amino acid composition using a Beckman amino acid analyzer. The purity was 80% by high-pressure liquid chromatography using a Perkin-Elmer instrument equipped with a C18 reverse-phase column. The peptide was conjugated to a mouse albumin carrier by the glutaraldehyde method (34). BALB/c mice were immunized with 10 µg of peptide-carrier/injection/mouse. The first shot (i.p.) was with complete Freund's adjuvant. The second injection, 2 wk later, was s.c. with incomplete Freund's adjuvant. All subsequent injections were s.c. without adjuvant within 2-wk intervals. Mice were bled prior to each injection, and serum titers of anti-N-myc peptide were determined by ELISA using plates (ELISA plates; Costar, Cambridge, MA) coated with pure N-myc peptide. Titers of up to 1:10,000 were obtained after 2 to 3 mo of immunization. Hybridomas were generated by fusing BALB/c splenocytes with SP2/O mouse myeloma cells as described before (35). Screening of positive cells was done by ELISA as was done for determination of serum titers. Alkaline phosphatase-anti-mouse IgG was used as secondary antibody followed by p-nitrophenyl phosphate as a substrate. Plates were screened, and absorbance was determined by an ELISA reader (Model 450, Biorad). Positive wells were cloned twice by the limiting dilution method. Immunoglobulin isotyping was performed using the Amersham isotyping kit (Amersham, Buckinghamshire, England). The antibody was purified, and the amino acid sequence of the constant region was determined. The antibody was then used to detect the expression of N-myc and c-myc oncogenes using immunoprecipitation and Western blotting.

Table 1 Phenotyping of newly established NB cell lines

<table>
<thead>
<tr>
<th>Patient</th>
<th>Morphology</th>
<th>Doubling time (h)</th>
<th>5.1.H11 (N-CAM)</th>
<th>M340</th>
<th>UJ13A (N-CAM)</th>
<th>Thy-1</th>
<th>UJ127.11 (L-1)</th>
<th>UJ181.4</th>
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</thead>
<tbody>
<tr>
<td>E. C.</td>
<td>L/D*</td>
<td>43.1</td>
<td>22.5</td>
<td>26.3</td>
<td>9.0</td>
<td>87.0</td>
<td>1.2</td>
<td>ND</td>
</tr>
<tr>
<td>G. M.</td>
<td>L/D</td>
<td>46.7</td>
<td>2.5</td>
<td>74.6</td>
<td>7.0</td>
<td>98.8</td>
<td>4.3</td>
<td>12.3</td>
</tr>
<tr>
<td>G. J.</td>
<td>L/D</td>
<td>64.6</td>
<td>11.5</td>
<td>60.5</td>
<td>25.5</td>
<td>94.1</td>
<td>3.9</td>
<td>45.1</td>
</tr>
<tr>
<td>W. C.</td>
<td>L/D</td>
<td>45.4</td>
<td>12.1</td>
<td>8.7</td>
<td>23.1</td>
<td>96.7</td>
<td>ND</td>
<td>17.3</td>
</tr>
<tr>
<td>B. D.</td>
<td>S/D</td>
<td>80.0</td>
<td>6.1</td>
<td>65.8</td>
<td>12.1</td>
<td>97.8</td>
<td>9.7</td>
<td>27.6</td>
</tr>
<tr>
<td>S. F.</td>
<td>L/D</td>
<td>40.0</td>
<td>44.7</td>
<td>10.6</td>
<td>59.0</td>
<td>90.5</td>
<td>ND</td>
<td>81.6</td>
</tr>
<tr>
<td>P. T.</td>
<td>L/D</td>
<td>67.2</td>
<td>44.2</td>
<td>45.9</td>
<td>23.4</td>
<td>94.5</td>
<td>2.7</td>
<td>ND</td>
</tr>
<tr>
<td>G. T.</td>
<td>S/N</td>
<td>46.7</td>
<td>31.9</td>
<td>25.8</td>
<td>12.3</td>
<td>92.4</td>
<td>2.0</td>
<td>ND</td>
</tr>
<tr>
<td>W. S.</td>
<td>L/D</td>
<td>45.4</td>
<td>1.2</td>
<td>3.1</td>
<td>0.7</td>
<td>93.0</td>
<td>1.1</td>
<td>ND</td>
</tr>
<tr>
<td>Cell line</td>
<td>CHP-126</td>
<td>S/N</td>
<td>24.0</td>
<td>80.0</td>
<td>3.3</td>
<td>55.2</td>
<td>75.1</td>
<td>2.1</td>
</tr>
</tbody>
</table>

* L/D, large differentiated; ND, not determined; S/D, small differentiated; S/N, small nondifferentiated.
^ Numbers represent the percentage of positive cells.

Fig. 1. N-myc-specific amino acid peptide sequence 336–348 in exon 3. Peptide was synthesized, conjugated to albumin, and used to immunize mice as described in "Materials and Methods." Note that only 4 of 13 amino acids overlap with c-myc.
sham, United Kingdom). Clone 5C6 used throughout this study is an IgM antibody secreting about 10 µg/ml of antibody. Western blot analysis revealed specific binding of 5C6 anti-N-myc antibodies to a band with an approximate molecular weight (Fig. 2, Lanes 5 and 6) as described previously for polyclonal antisera (36) and slightly higher molecular weight than shown for NCMII 100 (Oncogene Sciences, Manhasset, NY) anti-N-myc monoclonal antibody (Lane 9; compare with Lanes 5 and 6).

Fig. 3 is a fluorescence micrograph of CHP-126 cells stained (in suspension) with antibodies to N-myc (A and C) and c-myc (B and D). While c-myc staining is exclusively nuclear (B and D), staining for N-myc shows a diffuse pattern, nuclear and cytoplasmic. This is evident whether the secondary antibody is conjugated with PE (red) or with FITC (green). In E and F are cells dually stained with antibodies to N-myc (red) and c-myc (green). It is interesting to note that, in a dividing cell (E, top cell), c-myc clearly stains the chromosomes, whereas the same cell stained with antibodies to N-myc shows diffuse staining. When stained CHP-126 cells were analyzed by flow cytometry, an mpc of 50.0 ± 6 was obtained, whereas leukemic cell lines such as KG-1, HL-60, Nalm-B, and CEM showed an mpc of 10 ± 3, 9 ± 3, 3 ± 1, and 6 ± 3 ± SD. These results clearly demonstrate preferential expression of N-myc in NB cells (see also Table 2).

Titration studies for N-myc and c-myc antibody binding indicate saturation around 75 to 100 µl of hybridoma culture supernatant equivalent to 0.5 to 1 µg of antibody/test (Fig. 4). Using the CHP-126 cell line, saturation binding of c-myc antibodies yields 19.3 mpc units, whereas for N-myc it is about 4 fold higher (Fig. 4). All further binding studies were done at antibody saturation (100 µl).

Specificity of 5C6 antibodies to N-myc was confirmed by blocking experiments with pure N-myc peptide. Thus, preincubation of 100 to 200 µg of pure peptide for 1 h at 37°C with hybridoma culture supernatant resulted in 80 to 90% inhibition of antibody binding. No inhibition of 5C6 antibody binding was observed when the c-myc peptide (amino acid 177-186) was used at comparable concentrations (results not shown).

Staining of Newly Established NB Cell Lines. Table 2 compares the data obtained from quantitation of the expression of N-myc and c-myc in the newly established lines to that from CHP-126 (50 copies of N-myc gene) and SK-N-SH (one copy of N-myc), both lines being established many years ago (16, 23). The results indicate coexpression of c-myc and N-myc oncogenes in all cell lines tested. The level of expression of N-myc protein is 3- to 16-fold higher than c-myc in the same cells (e.g., Patients W. S. and B. A.). Furthermore, the expression of N-myc varies dramatically in cells from various patients (mpc of 32 to mpc of 250). On the other hand, the variations in c-myc oncogene expression are much less pronounced (mpc of 13 to mpc of 32). All newly established cell lines expressed equal or higher levels of N-myc oncoprotein than the CHP-126 cell line which is known to express 50 copies of the N-myc gene (24).

DNA ploidy revealed 11 of 14 diploid tumors. Cell morphology also varied between cell lines, with no obvious pattern of N-myc level or DNA ploidy (Table 2; see also Fig. 5). All cell lines expressed one of the three neurofilament triplet proteins, the M, 145,000 neurofilament (NF-M), whereas the other two neurofilaments (NF-L and NF-H) were rarely expressed and at a low level (patients K. H. and G. T.; Table 2). High levels of the MDR-1 protein were observed in 6 of 22 cell lines tested.

The results obtained from 14 cell lines are presented in Table 2.

Localization of N-myc and c-myc by Electron Microscopy. Fig. 5 depicts the localization of N-myc and c-myc by light microscopy (A and C) and electron microscopy (B and D) level. While c-myc is localized exclusively to the nucleus [C (green fluorescence) and D (15-nm gold particle-conjugated antibodies)], staining of N-myc oncoprotein indicates diffuse, nuclear, and cytoplasmic localization [Fig. 5, A (red fluorescence) and B (15-nm gold particle-conjugated antibodies)]

DISCUSSION

Occult Tumor Cells in Bone Marrow of Patients in Complete Remission. In this paper, we describe a new methodology for isolating and establishing cell lines from bone marrow metastases of NB patients. Although all the patients described in this study were in complete clinical remission at the time of marrow harvest (POG Protocol 8844) and exhibit <0.5% NB cells by immunofluorescence staining, still as many as 35 x 10⁶ NB cells could be recovered from the samples following a purge of 300 to 400 ml of marrow. Extrapolating for total body marrow volume, the figure could go over 1 x 10⁹ NB cells, excluding tumor cells in other organs (37). This finding is rather important in view of previous publications linking occult tumor cells in bone marrow to relapse of disease in bone marrow-transplanted small cell lung carcinoma patients (38). According to our results 9 of 18 patient had >1 x 10⁷ tumor cells in the “purger.” Three of these patients died within 6 mo of transplantation. It is too early to correlate our findings to clinical outcome. These results emphasize the necessity for marrow purging, although not clinically proving it, as yet.
Fig. 3. Two-color immunofluorescence staining of CHP-126 cell line for N-myc and c-myc oncoproteins. For experimental details, see *Materials and Methods. A and C, staining for N-myc; B and D, staining for c-myc. Note the exclusive nuclear staining for c-myc versus diffuse nuclear and cytoplasmic staining for N-myc. Switching the secondary antibody from PE (red) to FITC (green) does not change the pattern of staining. E and F, the same cells dually stained for N-myc (E, red) and c-myc (F, green). Note the dividing cell on the top (E) clearly showing staining of chromosomes with anti-c-myc antibodies. Staining of the same cell for N-myc shows a diffuse pattern. x 800.

Of special interest is our finding that tumor cells from different patients express each of the 5 different NB surface antigens, to a different extent. These findings emphasize tumor heterogeneity and further justify the use of a cocktail of antibodies for purging of tumor cells.

Expression and Quantitation of c-myc and N-myc Oncoproteins. A methodology was also developed for simultaneous staining and quantitation of N-myc and c-myc oncoprotein expression in NB cell lines. Quantitation of dually stained cells was achieved using the two fluorochromes FITC (green) and PE (red) conjugated to anti-mouse IgG- and mouse IgM-specific secondary antibodies. This methodology is very sensitive and quantitative as indicated in Fig. 4. Fairly linear binding of c-myc and N-myc antibodies was observed using 10 to 15 µl of hybridoma culture supernatants reaching saturation at 100 to 200 µl of hybridoma supernatant. At saturation, c-myc binding reaches a maximum mpc of 19.3 units, whereas N-myc binding reaches a maximum of 62.6 units. These ratios remain similar when the fluorochromes are switched, indicating that the differences in staining are real (results not shown). Differences in the intensity of staining between N-myc and c-myc could be demonstrated using the immunogold labeling technique (Fig. 5, compare B to D). Over 90% of the cells are dually stained for c-myc and N-myc, confirming for the first time that c-myc and N-myc oncoproteins can be coexpressed in the same cell. All cell lines tested expressed both oncogenes (see, for comparison, Table 2). However, when leukemic cell lines were stained for c-myc and N-myc, staining for c-myc was 5- to 10-fold stronger than staining for N-myc oncoprotein. The promyelocytic leukemia cell line HL-60 which does not have detectable N-myc mRNA, and thus is used as a negative control in most publications (9, 39), showed modest expression of N-myc (mpc of 9 ± 3 units). Interestingly, the NB cell line SK-N-SH, with one copy of the N-myc gene and no detectable mRNA to N-myc, showed an appreciable amount of N-myc protein expression, implying that the immunofluorescence staining of N-myc
ONCOGENES AND MDR-1 EXPRESSION IN NEW NEUROBLASTOMA CELL LINES

Fig. 4. Flow cytometric titration of antibodies to N-myc and c-myc oncoprotein coexpressed in the CHP-126 cell line. For experimental details, see “Materials and Methods.” Cells were stained for both N-myc and c-myc. Anti-mouse IgM-PE was used to detect the N-myc antibody (5C6, mouse IgM), and anti-mouse IgG-FITC (Fcγ specific) was used to detect the anti-c-myc antibody (mouse IgG). The mpc was used to quantitate fluorescence intensity. Unimodal distribution of stained cells with a coefficient of variation of 4 to 6% was consistently observed.

protein is more sensitive than Northern blot analysis of mRNA to N-myc (9).

Expression of N-myc Protein in NB patients. The expression of N-myc oncoprotein varied greatly among the patients tested with 10 of 13 patients tested expressing elevated N-myc protein (mpc > 50 units), six of which died within 6 mo posttransplantation. Furthermore, 3 of 3 patients with a low level of N-myc protein (mpc < 50 units) remained disease free 6 to 12 mo posttransplantation (Table 2). Thus, it seems that measurement of the N-myc protein level might have a better correlation to the patient’s relapse than measurement of gene copy number amplification, which correlates only with 30% of relapsing cases (1–10). Our findings are in agreement with a case report published by Cohn et al. (9) in which it was demonstrated that N-myc protein levels determined by Western blot analysis correlated better with adverse prognosis in a patient with the non-amplified N-myc gene.

Detection of N-myc oncoprotein in NB cells metastasizing to bone marrow by polyclonal antiserum against the N-myc fragment (bGH/N-myc II) and staining with immunoperoxidase was described before (40). In their studies, Moss et al. used patients’ marrow at diagnosis with over 10% NB cells in the marrow. Qualitative estimates of N-myc protein (on a scale of 0 to 3) was compared to gene copy number in the same six patients. No correlation was observed between N-myc protein and gene copy number. Furthermore, the clinical value of the N-myc protein expression versus gene copy number was not shown. The small number of patients tested, the use of polyclonal antibodies, and the lack of quantitation make it difficult to evaluate these results. Correlation between N-myc gene copy number and protein expression was reported recently by Hashimoto et al. (8). In this study N-myc protein was detected by immunoperoxidase staining using rabbit polyclonal anti-N-myc antibody. Staining for N-myc was evaluated on a

Table 2 Characterization of newly established neuroblastoma cell lines

<table>
<thead>
<tr>
<th>Patient</th>
<th>N-myc mpc</th>
<th>c-myc (%) positive</th>
<th>DNA index</th>
<th>Morphology</th>
<th>Neurofilaments</th>
<th>MDR-1 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Low</td>
<td>Medium</td>
</tr>
<tr>
<td>W. S.</td>
<td>32.1 (79)</td>
<td>18.3 (81)</td>
<td>1.1</td>
<td>L/D</td>
<td>±</td>
<td>+3</td>
</tr>
<tr>
<td>G. T.</td>
<td>86.0 (81)</td>
<td>32.0 (86)</td>
<td>1.0</td>
<td>S/N</td>
<td>+2</td>
<td>+4</td>
</tr>
<tr>
<td>E. C.</td>
<td>64.9 (92)</td>
<td>22.1 (98)</td>
<td>2.1</td>
<td>L/D</td>
<td>−</td>
<td>+3</td>
</tr>
<tr>
<td>G. M.</td>
<td>204.0 (86)</td>
<td>28.1 (81)</td>
<td>2.0</td>
<td>L/D</td>
<td>−</td>
<td>+4</td>
</tr>
<tr>
<td>B. D.</td>
<td>ND</td>
<td>ND</td>
<td>1.8</td>
<td>L/D</td>
<td>−</td>
<td>+3</td>
</tr>
<tr>
<td>G. J.</td>
<td>78.4 (91)</td>
<td>12.8 (90)</td>
<td>2.1</td>
<td>L/D</td>
<td>−</td>
<td>+4</td>
</tr>
<tr>
<td>B. A.</td>
<td>249.7 (96)</td>
<td>15.0 (82)</td>
<td>1.9</td>
<td>L/D</td>
<td>±</td>
<td>+3</td>
</tr>
<tr>
<td>W. C.</td>
<td>43.0 (61)</td>
<td>18.0 (84)</td>
<td>0.95</td>
<td>L/D</td>
<td>±</td>
<td>+3</td>
</tr>
<tr>
<td>S. F.</td>
<td>112.1 (94)</td>
<td>22.2 (90)</td>
<td>1.1</td>
<td>L/D</td>
<td>−</td>
<td>+3</td>
</tr>
<tr>
<td>H. K.</td>
<td>76.9 (98)</td>
<td>12.6 (80)</td>
<td>1.2</td>
<td>L/D</td>
<td>−</td>
<td>+2</td>
</tr>
<tr>
<td>L. J.</td>
<td>67.0 (83)</td>
<td>20.3 (92)</td>
<td>1.3</td>
<td>L/D</td>
<td>−</td>
<td>+2</td>
</tr>
<tr>
<td>P. T.</td>
<td>89.5 (94)</td>
<td>16.8 (96)</td>
<td>2.1</td>
<td>L/D</td>
<td>−</td>
<td>+2</td>
</tr>
<tr>
<td>M. F.</td>
<td>73.0 (78)</td>
<td>14.1 (48)</td>
<td>1.0</td>
<td>L/D</td>
<td>−</td>
<td>+2</td>
</tr>
<tr>
<td>G. A.</td>
<td>36.0 (81)</td>
<td>16.5 (78)</td>
<td>1.1</td>
<td>L/D</td>
<td>±</td>
<td>+2</td>
</tr>
</tbody>
</table>

Patients died.

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ONCOGENES AND MDR-1 EXPRESSION IN NEW NEUROBLASTOMA CELL LINES

Fig. 5. Ultrastructure localization of N-myc and c-myc oncoproteins in the neuroblastoma cell line from patient G. J. For experimental details, see "Materials and Methods." A and B and C and D are cells stained for N-myc and c-myc, respectively. Note the diffuse pattern of N-myc staining shown in both the fluorescence micrograph (A) and the electron micrograph (B). Gold particles (15 nm) are clearly visible in the nucleus (N), along the inner side of the nuclear membrane, and in the cytoplasm, although to a lesser extent. The arrow indicates the nuclear membrane. c-myc oncoprotein (C and D), on the other hand, is restricted to the nucleus. Fewer gold particles are seen in the nucleus stained for c-myc (D) as compared to N-myc staining (B).

In general, N-myc protein expression correlated in 13 of 18 patients tested. Five patients whose N-myc gene was amplified (6- to 37-fold) had little or no staining for N-myc protein. However, most patients with a high copy number and a high level of N-myc protein died within 6 to 12 mo, whereas patients with no gene amplification and no detectable N-myc protein were alive 2 yr or more (8). According to our data 10 of 13 patients had elevated N-myc protein (mpc > 50.0), six patients of which died within 6 mo. Although our results indicate good correlation between disease progression and expression of N-myc protein, it is important to bear in mind that a relatively small number of patients was analyzed with relatively short follow-up. Also, in spite of our effort to determine the levels of N-myc protein as close to initiation of cultures as possible, there is always the possibility that the majority of cells obtained by in vitro expansion will not represent all tumor cells in vivo. Studies including detailed comparison of gene copy number, mRNA level, and protein level of N-myc and c-myc in our newly established NB lines are in progress.

Expression of MDR-1 Protein and DNA Ploidy. The expression of the multidrug resistance (MDR-1) mRNA at diagnosis has also been implicated in the prognosis of NB patients (22). Our results indicate that, although the patients were in clinical remission at the time of staining for the MDR-1 protein, 6 of 22 were found to be positive for MDR-1 protein, two of which died within 6 mo. Follow-up of these patients will be important in determining the clinical value of the MDR-1 protein expression in predicting relapse. Interesting results pointing at inverse correlation between MDR-1 mRNA expression and N-myc mRNA expression were reported recently, with low levels of mRNA for MDR-1 in patients who became drug resistant following chemotherapy. These same patients had high levels...
of mRNA to N-myc (41). Thus, the importance of MDR-1 expression in NB remains unclear at this point.

DNA ploidy was also reported to be a prognosticator for disease progression in NB patients, although less than the N-myc amplification (7, 18–20). In our studies, 8 of 13 patients were near diploid, two of which died within 6 mo, with heterogeneous expression of the N-myc oncprotein (Table 2).

Localization of N-myc and c-myc. c-myc is thought to be localized to the nucleus (42), and microinjected c-myc protein migrates rapidly to the nucleus with a specific segment (amino acid 320–328) for signaling translocation to the nucleus (43). Yet, cytoplasmic distribution of c-myc was recently reported in human colorectal neoplasms, compared to nuclear localization for nonneoplastic mucosa (44). Although less studied, localization of the N-myc oncprotein in NB cell lines by immunoperoxidase and immunofluorescence staining with different anti-N-myc antibodies indicated nuclear staining (39, 45). Nevertheless, small quantities of N-myc protein could be immunoprecipitated from cytoplasmic fractions of NB cell lines (36, 45). Similar to c-myc, N-myc was also shown to have a nucleus translocation signal peptide (amino acid 345–353) (43), of which four amino acids (Pro-Pro-Gln-Lys) overlap with the translocation signal peptide (amino acid 345–353) (43), of which four amino acids (Pro-Pro-Gln-Lys) overlap with the translocation signal peptide (amino acid 345–353) (43), of which four amino acids (Pro-Pro-Gln-Lys) overlap with the translocation signal peptide (amino acid 345–353) (43), of which four amino acids (Pro-Pro-Gln-Lys) overlap with the translocation signal peptide (amino acid 345–353) (43), of which four amino acids (Pro-Pro-Gln-Lys) overlap with the translocation signal peptide (amino acid 345–353) (43), of which four amino acids (Pro-Pro-Gln-Lys) overlap with the translocation signal peptide (amino acid 345–353) (43), of which four amino acids (Pro-Pro-Gln-Lys) overlap with the translocation signal peptide (amino acid 345–353) (43), of which four amino acids (Pro-Pro-Gln-Lys) overlap with the 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four amino acids (Pro-Pro-Gln-Lys) overlap with the translocation signal peptide (amino acid 345–353) (43), of which four amino acids (Pro-Pro-Gln-Lys) overlap with the translocation signal peptide (amino acid 420–430) (43), of which four amino acids (Pro-Pro-Gln-Lys) overlap with the translocation signal peptide (amino acid 345–353) (43), of which four amino acids (Pro-Pro-Gln-Lys) overlap with the translocation signal peptide (amino acid 345–353).
ONCOGENES AND MDR-1 EXPRESSION IN NEW NEUROBLASTOMA CELL LINES


Expression of N-myc, c-myc, and MDR-1 Proteins in Newly Established Neuroblastoma Cell Lines: A Study by Immunofluorescence Staining and Flow Cytometry

Yair Gazitt, Yun Ju He, Lee Chang, et al.


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