Sensitive Detection of Rare Circulating Neuroblastoma Cells by the Reverse Transcriptase-Polymerase Chain Reaction

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ABSTRACT

The presence of circulating tumor cells in patients with localized or disseminated neuroblastoma may be a significant prognostic factor at diagnosis and may antedate the detection of relapse by other diagnostic studies. We report the development of a highly sensitive detection assay for circulating neuroblasts based on the reverse transcriptase-polymerase chain reaction (RT-PCR), using the neuroendocrine protein gene product 9.5 (PGP 9.5) as the tumor marker. Analysis of RT-PCR products by agarose gel electrophoresis demonstrated that neuroblastoma cell lines were uniformly positive, whereas peripheral blood mononuclear cells were negative. Alkaline Southern blotting with a PGP 9.5-specific probe revealed scant expression of PGP 9.5 in peripheral blood mononuclear cells, well below the limits of detection by electrophoresis alone. The system was able to detect a single neuroblastoma cell in 10^7 peripheral blood mononuclear cells. Eighteen patient samples were analyzed by PGP 9.5 RT-PCR and the results compared with immunocytochemistry in 16. Ten of the 18 were negative by both studies. Eight of the 18 were positive by PGP 9.5 RT-PCR, 4 of which were also positive by immunocytochemistry. PGP 9.5 RT-PCR was able to detect circulating neuroblasts in two patients with negative immunocytochemistry, the first with progressive bone marrow disease and the second at high risk for relapse. No other evidence of disease. PGP 9.5 RT-PCR allows the detection of circulating neuroblastoma cells with a sensitivity greater than immunocytochemistry. It will be useful in evaluating the clinical significance of circulating tumor cells with respect to prognosis and early detection of relapse. and in the screening of peripheral stem cell harvests prior to autologous infusion.

INTRODUCTION

Neuroblastoma is the most common extracranial solid tumor of childhood, with an annual incidence of 9.4 per million children under 15 years of age (1). While the prognosis of this malignancy has improved with advances in medical management, overall 5-year survival rate is currently only 55% (1). Prognostic factors significant in delineating subgroups of patients with differing outcomes include age, stage, serum ferritin level, histopathological features, tumor cytogenetic findings, tumor DNA content, and level of expression and copy number of the protooncogene N-myc in tumor cells (2-5). Recent studies have demonstrated that the immunocytochemical detection of rare neuroblasts in bone marrow predicts clinical outcome in relation to age at diagnosis (6), and that the presence of circulating neuroblasts during treatment correlates with tumor relapse (7).

Given the importance of rare disseminated tumor cells in the prognosis of neuroblastoma patients, we sought to develop a sensitive assay for the detection of circulating neuroblastoma cells. We now report the development and application of such an assay by using a highly sensitive RT-PCR amplifying RNA transcribed by the neuronal gene PGP 9.5. This RT-PCR system has a sensitivity 100-fold greater than immunocytochemistry, while retaining specificity for tumor cells. Patient samples have been analyzed and the results correlated with immunocytochemical and clinical data. This technique is directly applicable to the diagnostic assessment of patients with neuroblastoma, and will be useful in the early detection of relapse.

MATERIALS AND METHODS

Neuroblastoma Cell Lines. SKNMC and SKNSH were purchased from the American Type Culture Collection (Rockville, MD), and cultured in minimum essential medium (Gibco, Grand Island, NY) with 10% heat-inactivated fetal calf serum. LAN-2 and LAN-5 were purchased from Robert Seeger, MD (Children's Hospital of Los Angeles), and cultured in RPMI 1640 (Gibco) with 10% heat-inactivated fetal calf serum.

Blood and Bone Marrow Samples. Normal blood and bone marrow samples were obtained from volunteers. Patient blood samples were obtained at diagnosis, during treatment, or at relapse. PBMNC and BMMNC were isolated by Ficoll-Paque (Pharmacia, Uppsala, Sweden) density centrifugation. Some PBMNC were stored in medium containing 10% dimethyl sulfoxide under liquid nitrogen and subsequently analyzed.

RNA Extraction. Total cellular RNA was extracted from tumor cell lines, BMMNC, and some normal PBMNC samples by a modification of the guanidinium thiocyanate/cesium chloride method (8) (method A), and diluted to a final concentration of 1 µg/µL DEPC-treated water. To extract total cellular RNA from small numbers of cells (0.5 to 10^7), the following modified protocol was used (method B). Cells were centrifuged in 1.5 ml Eppendorf tubes at 15,000 x g. The cell pellet was lysed in 500 µl 4 x guanidinium thiocyanate (BRL, Gaithersburg, MD), 25 mM citrate, pH 7.0, 0.5% n-lauryl sarcosine solution, vortexed, and placed at -70°C for 1 h. The lysate was thawed, 250 µl 100 mM Tris, pH 8.0, 10 mM EDTA, 1% sodium dodecyl solution were added, and this solution was extracted twice with equal volumes of phenol:chloroform:2% isoamyl alcohol, then twice with chloroform:2% isoamyl alcohol. RNA visible at the interface was removed directly by using a pipet. The aqueous phase was divided into two 1.5-ml Eppendorf tubes; 10 µg rRNA, one tenth volume 3 M sodium acetate, pH 5.2, and 2.5 volumes absolute ethanol were added, and the samples were precipitated 1 h at -70°C. The samples were thawed, centrifuged at 15,000 x g for 20 min, washed with 70% ethanol, dried, and resuspended in DEPC-treated water to a final volume such that 10 µL of the RNA suspension represented 10^6 cells in the original pellet.

Reverse Transcriptase-Polymerase Chain Reaction. All oligonucleotides were synthesized by the University of Michigan Molecular Genetics Center (Table 1). The RT reaction mixture consisted of 5 µL of RNA prepared by method A, or 10 µL of RNA prepared by method B, and DEPC-treated water to a final volume such that 10 µL of the RNA suspension represented 10^6 cells in the original pellet.

Received 2/21/92; accepted 6/17/92.

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1 This research was supported by research awards from the United States NIH.

2 Recipient of a Scholar Award from the Leukemia Society of America. To whom requests for reprints should be addressed, at Room 5510B MSRB1, 1150 West Medical Center Drive, Ann Arbor, MI 48109-0680.
water to a total volume of 50 µl. One hundred units MMV reverse transcriptase (BRL) were added, and the reaction was allowed to proceed at 41°C for 1 h. The PCR mixture consisted of 5 µl 10 × Taq RT buffer (400 mM KCl, 0.1% gelatin), 5 µl 1 mM deoxynucleotide triphosphate, 0.5 µg each primer, 10 µl RT product, in a total volume of 50 µl. Three units AmpliTaq (Perkin Elmer Cetus, Norwalk, CT) were added and solution was overlaid with mineral oil. The PCR conditions were: 40 s at 94°C, 75 s at 72°C, for 35 cycles, followed by a 10-min extension at 72°C. For each sample, 13.5 µl were electrophoresed on a 3% agarose gel ([2% NuSieve GTG agarose (FMC BioProducts, Rockland, ME) plus 1% standard agarose (International Biotechnologies, New Haven, CT)] and visualized by ethidium bromide staining. 32P RT DNA/HaeIII was purchased from BRL.

Alkaline Southern Analysis. Agarose gels were transferred overnight onto Zeta-Probe (Bio-Rad, Richmond, CA) nylon membrane with the use of a 0.4 N NaOH buffer, by an established procedure (8). 32P-End-labeled oligonucleotide probe was added (specific activity, 2.5 × 106 dpm) and allowed to hybridize at 37°C overnight. The membrane was washed using a 6 × standard saline citrate, 0.1% sodium dodecyl sulfate solution, and bands were visualized by autoradiography.

Immunocytological Analysis. Patient PBMCN and BMNC samples were prepared as above. Peripheral blood stem cells were collected by using the Fenwal CS3000, as previously described (9). Mononuclear cells were resuspended in Liebowitz L-15 medium with 10% fetal calf serum at a dilution of 104 cells/ml and incubated at room temperature for 45 min with a mixture of 4 monoclonal antibodies directed against fetal brain, N-CAM, GPr, and Thy-1 (monoclonal antibodies 459, 390, HSAN 1.2, 126-4, 14-2A, UJ13A, 5.1.H.11) (10) at a concentration of 5 µg/antibody/106 cells. The cells were washed twice with L-15/10% fetal calf serum, cytocentrifuged onto coverslips (75,000 cells/coverslip), and stained by using the avidin biotin-peroxidase technique as previously described (11).

RESULTS

Specificity: Expression of PGP 9.5 in Normal PBMCN, Normal BMNCN, and Tumor Cell Lines. PGP 9.5 is a 212 amino acid, Mr 24,500 ubiquitin carboxyl-terminal hydrolase (12) which constitutes 1–2% of total soluble brain proteins (13), where it is localized exclusively in neuronal cytoplasm (14). Immunohistochemical studies have demonstrated the human tissue distribution also to include retina, peripheral neurons and axons, anterior pituitary, melanocytes, thyroid parafollicular cells, pancreatic islets, gastric endocrine cells, adrenal medullary cells, and fetal Kulitschzky and bronchial epithelial cells (13). The complementary DNA sequence and genomic organization of the PGP 9.5 gene have been reported (15, 16). Oligonucleotides (17) for RT-PCR amplification from mRNA (Table 1) included a PGP 9.5-specific RT primer (A: nucleotides +737 to +723), and PCR primers designed to span exon splice junctions (B: nt -13 to +18, C: nt +640 to +607). Oligonucleotide D (nt +256 to +288), internal to B and C, was used as the alkaline Southern probe.

PGP 9.5 RT-PCR of total cellular RNA from four neuroblastoma cell lines (LAN-2, LAN-5, SKNSH, and SKNMC) demonstrated amplification (Fig. 1) of a ~650-nt fragment (expected product length, 653 nt). Alkaline Southern transfer and probing with 32P-end-labeled oligonucleotide D confirmed the identity of the fragment as PGP 9.5 (results for SKNMC shown in Fig. 2). Two additional neuroblastoma cell lines (LAN-1, KCNR) were tested and were likewise positive (data not shown).

We then assayed PBMCN and BMNCN obtained from normal donors. Agarose gel analysis of the amplification products (Fig. 2A) showed no detectable 653 nt bands. Alkaline Southern blotting using the PGP 9.5-specific oligonucleotide D showed faint positivity at 653 nt for PBMCN and stronger positivity for BMNCN (Fig. 2B). Thus, these normal cell populations express scant amounts of PGP 9.5 mRNA, which are below the level of detection of the assay system as analyzed by electrophoresis, and which are not sufficient to confer false positivity. In our hands, reverse transcription of total cellular RNA followed by a single round (35 cycles) of PCR gave reliable results without evidence of RNA carryover contamination.

Sensitivity Testing. We used two strategies, serial dilution and limiting dilution, to determine assay sensitivity. First, sensitivity was determined by performing serial dilutions of SKNMCN cells and preparing mixes with PBMCN to represent 100, 10, and 1 tumor cell(s)/107 PBMCN. The SKNMCN cell line was selected for this experiment because of its ability to be diluted to single-cell suspensions without clumping. PGP 9.5 mRNA was detected at concentrations as low as 1 tumor cell/107 PBMCN, although the degree of amplification was not quantitative (Fig. 3). To verify the detection sensitivity of 1 tumor cell in 107 PBMCN, we prepared 10 sample mixes at that dilution, isolated the RNA, and performed RT-PCR. One of three such experiments is shown in Fig. 4. Poisson limiting dilution statistics would predict that 70% of such mixes would contain a single tumor cell, whereas 30% would not contain a tumor cell. The results are consistent with the Poisson prediction, with 8 of 10 lanes demonstrating amplification.

Patient Samples. To determine whether this system could detect neuroblastoma cells in clinical samples and to correlate the findings with immunocytology, we analyzed peripheral blood specimens obtained from 18 patients with neuroblastoma. Amplifications from peripheral blood cells from three of these patients, two of whom were positive for PGP 9.5, are shown in Fig. 5. Table 2 presents data from the eight patients whose PB samples had neuroblasts detected by PGP 9.5 RT-PCR. Of those evaluated at diagnosis, patient 1 (stage III), and patients 2 and 3 (stage IV with BM involvement) had detectable
DETECTING RARE CIRCULATING NEUROBLASTS

PBMNC BMMNC SKNMC CNTRLS

CD O5 O5 O5
QL Q_ Q_ Q_
Ni CD S CD S CD S CD S

Fig. 2. PGP 9.5 RT-PCR and alkaline Southern blot of normal PBMNC, BMMNC, and SKNMC. A, total cellular RNA was prepared according to method A (see text) from PBMNC and BMMNC obtained from normal donors, and from the SKNMC cell line. One µg RNA was used per RT reaction. RT-PCR was performed as described in text. One-fifth of PCR product volume (10 µl) was loaded per lane. Positive controls (CNTRLS) for each RNA preparation used RT and PCR primers specific for the constitutively expressed abl gene, with expected amplified fragment length of 235 nt. Control PCR reactions performed using all of the reagents as for the experimental samples, but without added RNA, yielded no signals (final two lanes). PGP 9.5 amplifications from 10 additional donors were also negative (data not shown). B, the gel from A was transferred onto nylon under alkaline conditions, as described in text. The PGP 9.5-specific 32P-end-labeled oligonucleotide D was hybridized overnight and washed with 6x standardsaline citrate/0.1% sodium dodecyl sulfate for 30 min at 65°C. The autoradiogram was exposed for 45 min.

PGP 9.5
abl

Fig. 3. Sensitivity testing: serial dilution. Serial dilution of the SKNMC cell line was used to prepare a solution of 1 cell/0.5 ml. PBMNC were diluted to a concentration of 10^7/ml. Ten separate cell mixes (1-10) were made by using 0.5 ml SKNMC suspension and 1.0 ml PBMNC suspension. Total cellular RNA from each mix was prepared according to method B (see text). RT-PCR was performed on each of these samples, and on a control (CNTL) sample without added RNA, as in Fig. 3. One-fifth of PCR product volume (10 µl) was loaded per lane. Positive PGP 9.5 amplimers were visible in Lanes 2, 3, 4, 5, 6, 7, 8, and 10, but not in Lanes 1 or 9. Positive controls for each RNA preparation (abl) were as in Fig. 2.

PGP 9.5
abl

Fig. 4. Sensitivity testing: limiting dilution. Serial dilution of the SKNMC cell line was used to prepare a solution of 1 cell/0.5 ml. PBMNC were diluted to a concentration of 10^7/ml. Ten separate cell mixes (1-10) were made by using 0.5 ml SKNMC suspension and 1.0 ml PBMNC suspension. Total cellular RNA from each mix was prepared according to method B (see text). RT-PCR was performed on each of these samples, and on a control (CNTL) sample without added RNA, as in Fig. 3. One-fifth of PCR product volume (10 µl) was loaded per lane. Positive PGP 9.5 amplimers were visible in Lanes 2, 3, 4, 5, 6, 7, 8, and 10, but not in Lanes 1 or 9. Positive controls for each RNA preparation (abl) were as in Fig. 2.

PGP 9.5
abl

Fig. 5. Detection of PGP 9.5 RNA from peripheral blood patient samples. Total cellular RNA was isolated by method B (see text) from 5 ml peripheral blood from 3 neuroblastoma patients with no evidence of disease at the time of study (Lanes 1-3). RT-PCR was performed on each of these samples, and on a control (CNTL) sample containing no added RNA. One-fifth of PCR product volume (10 µl) was loaded per lane. Positive PGP 9.5 amplimers were visible in Lanes 1-3. No amplimers were visible in CNTL. PGP 9.5 amplimers were also visible in Lanes 4-6, but not in Lanes 7-9. Positive controls for each RNA preparation (abl) were as in Fig. 2.

DISCUSSION

Neuroblastoma cells in the peripheral blood and bone marrow of patients at diagnosis and relapse have traditionally been detected by histological review. More recently, immunoperoxidase staining utilizing a combination of monoclonal antibodies directed against tumor surface antigens has increased the detection sensitivity to approximately 1-2 cells/10^5 mononuclear cells (6, 7). Immunocytological studies have shown that neuroblasts were present in BM samples from 34% of patients with circulating neuroblasts confirmed by immunocytology; patient 4 had stage IV-S disease with an adrenal primary, massive hepatic metastases, and immunocytological detection of BM involvement (PB immunocytology was not available for comparison). Patient 5 developed progressive BM disease while on chemotherapy, with detectable circulating neuroblasts by immunochemistry. Patient 6 relapsed in the BM subsequent to BMT, with no detectable PB neuroblasts by immunocytology. Patients 7 and 8 were assessed at 4 and 2 months, respectively, following BMT, and were disease free at the time of sampling by conventional and immunocytological testing (further follow-up is not yet available); however, both patients had immunocytological evidence of disease at the time of transplant (patient 7, PB; patient 8, BM). Ten additional patients with neuroblastoma were tested for the presence of circulating neuroblasts by both PGP 9.5 RT-PCR, immunocytology, and histology; in all cases, these methods yielded concordant results, with no neuroblasts detected. Taken together, these results suggest that PGP 9.5 RT-PCR is more sensitive than immunocytochemistry for the detection of circulating neuroblastoma cells.

Fig. 5. Detection of PGP 9.5 RNA from peripheral blood patient samples. Total cellular RNA was isolated by method B (see text) from 5 ml peripheral blood from 3 neuroblastoma patients with no evidence of disease at the time of study (Lanes 1-3). RT-PCR was performed on each of these samples, and on a control (CNTL) sample containing no added RNA. One-fifth of PCR product volume (10 µl) was loaded per lane. Positive PGP 9.5 amplimers were visible in Lanes 2, 3, 4, 5, 6, 7, 8, and 10, but not in Lanes 1 or 9. Positive controls for each RNA preparation (abl) were as in Fig. 2.

PGP 9.5
abl

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localized or regional disease (stages I, II, and III), which correlated with decreased progression-free survival in select populations (6); that neuroblasts were present in the PB of three patients on therapy prior to evidence of progressive disease by conventional studies (7); and that a substantial portion (25%) of peripheral stem cell harvests from patients with disseminated disease had neuroblasts contaminating the harvested cells (11). Therefore, sensitive methods allowing earlier diagnosis of relapse, detection of contaminating neuroblasts in peripheral stem cell harvests prior to autologous infusion, and documenting minimal residual disease at completion of therapy or prior to BMT are essential in order to tailor therapies for defined patient populations to achieve an increase in patient survival.

In the present study, PGP 9.5 mRNA expression has been used as a marker for the presence of circulating neuroblasts. Whereas a number of malignancies, including chronic myelogenous leukemia, are characterized by discrete and consistent chromosomal translocations which can be exploited for purposes of tumor detection (17, 18), the known cytogenetic and molecular genetic abnormalities associated with neuroblastoma tumor cells do not lend themselves to such application. Specifically, although dysregulation of the protooncogene N-myc is strongly implicated in the pathogenesis of neuroblastoma (19–22), its overexpression and/or amplification does not provide a basis for diagnostic testing. Similarly, the presence of partial deletions, point mutations, and loss of heterozygosity (4) involving chromosome 1p may be associated with tumorogenesis, but do not constitute a reliable genetic marker for tumor detection. PGP 9.5 has a tissue distribution virtually restricted to neural crest cells. All neuroblastoma cell lines and de novo tumor cells tested by RT-PCR have expressed PGP 9.5. Such uniform expression of PGP 9.5 among tumor cells makes this gene an ideal candidate as a marker for use in RT-PCR. Whether PGP 9.5 plays a role in tumorigenesis or whether its expression reflects high tumor cell metabolism is not known, but remains to be explored.

Normal PBMC and BMMNC were analyzed by RT-PCR and agarose gel electrophoresis for the expression of PGP 9.5. These cell populations were negative, indicating that this assay would be useful for the detection of contaminating neuroblasts. Although alkaline Southern blotting subsequently demonstrated scant amounts of PGP 9.5 expression, this very low level of expression has not conferred false positivity on the assay system when applied to PB. Further normal BM and patient samples will be assayed to assess the usefulness of the system for BM screening.

We have demonstrated that PGP 9.5 RT-PCR has the ability to detect one neuroblastoma cell in 10^7 normal PBMC, as shown by both serial dilution and limiting dilution (Figs. 3 and 4). Assay sensitivity is reflective of the mRNA copy number present in the tumor cells. The guanidinium isothiocyanate RNA isolation technique used for clinical samples allowed for the analysis of small PB sample volumes (5 ml) easily obtained from pediatric patients. Consistency in the amount of total cellular RNA used in the RT reaction was obtained by using an amount of RNA suspension (10 µl) representing 10^6 cells in the original sample. An aliquot of this suspension used in the RT reaction is sufficient to screen the entire initial sample for the presence of tumor cells.

The clinical applicability of PGP 9.5 RT-PCR was documented by evaluating 18 patient PB samples. Of these, 10 did not have circulating neuroblasts by histological or immunocytochemical assays, and were also negative by our system. In these situations, PGP 9.5 RT-PCR contributes confirming data with higher sensitivity. The remaining eight patients (Table 2) had detectable circulating neuroblasts by RT-PCR. In four, PB immunocytochemistry was positive, demonstrating concordance between assays. One of these patients was stage III, revealing that regional disease can be accompanied by circulating tumor cells. In a fifth patient (stage IV-S), PB immunocytochemistry was not available for comparison; however, a BM sample obtained simultaneously was positive by immunocytochemistry. The final three patients illustrate the potential utility of the increased sensitivity provided by our system. Patients 6 and 7 had detectable circulating neuroblasts by PGP 9.5 RT-PCR undetected by immunocytochemistry. Patient 8 had BM tested by immunocytochemistry which was negative; PB immunocytochemistry was not available for comparison. Patient 6 had histologically proven BM disease at the time of sampling, illustrating in a clinical setting that PGP 9.5 RT-PCR has higher sensitivity for circulating tumor cells than immunocytochemistry. Patients 7 and 8 had no other identifiable disease at the time; however, both had immunocytologically detectable disease at the time of BMT (patient 7, PB; patient 8, BM) and thus may be at high risk for relapse in the future.

The 100-fold increase in sensitivity provided by PGP 9.5 RT-PCR compared to immunocytochemistry will prove useful in evaluating the clinical significance of circulating neuroblasts at diagnosis, and may identify subgroups of patients with low-stage disease requiring more intensive therapy. Future applications will include the monitoring of patients to determine the ability of the assay to predict relapse at earlier timepoints, and in screening peripheral stem cell harvests for evidence of contaminating tumor cells prior to autologous infusion. Overall, while PGP 9.5 RT-PCR appears to be a more sensitive technique than immunohistochemistry for analyzing peripheral blood specimens, the interpretation of such data must be verified through the analysis of more patients and longer periods of follow-up clinical data.

### Table 2 Clinical data on patients with positive circulating PGP 9.5 RT-PCR assays

<table>
<thead>
<tr>
<th>Patient</th>
<th>Stage at diagnosis</th>
<th>Disease Status at time of sampling</th>
<th>No. of Immunocytology (NBL cells/10^5 MNC)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>BM</td>
</tr>
<tr>
<td>1</td>
<td>III</td>
<td>At diagnosis</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>IV: abd, bone, BM</td>
<td>At diagnosis</td>
<td>N/D</td>
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<tr>
<td>3</td>
<td>IV: abd, BM</td>
<td>At diagnosis</td>
<td>36,501</td>
</tr>
<tr>
<td>4</td>
<td>IV-S: adrenal, liver</td>
<td>At diagnosis</td>
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</tr>
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<td>5</td>
<td>IV: abd, bone, BM</td>
<td>Progressive: soft tissue, bone</td>
<td>9</td>
</tr>
<tr>
<td>6</td>
<td>IV: abd, bone, BM</td>
<td>Progressive: BM</td>
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<tr>
<td>7</td>
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<td>No evidence of disease</td>
<td>N/D</td>
</tr>
<tr>
<td>8</td>
<td>IV: abd, bone, BM</td>
<td>No evidence of disease</td>
<td>0</td>
</tr>
</tbody>
</table>

Abbreviations: NBL, neuroblastoma; MNC, mononuclear cells; abd, abdominal; N/D, not done.
ACKNOWLEDGMENTS

We would like to thank Drs. Lisa R. Gottschalk and Susan S. Mattano for their expert technical advice and helpful suggestions, and Martha Davis for her expert secretarial assistance.

REFERENCES

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