Shedding of membrane gangliosides is characteristic of human and experimental tumors. Because some shed tumor gangliosides have potent tumor-enhancing properties, significant ganglioside shedding could influence tumor progression. We examined this possibility in a human tumor, neuroblastoma. Ganglioside shedding, measured as circulating tumor-derived G02 ganglioside, and the outcome of 74 patients with advanced stage (III and IV) disease were studied. Progression-free survival (PFS) was inversely related to circulating G02 levels at the time of diagnosis (P = .018). By Kaplan-Meier analysis, the quartile of patients having the highest circulating G02 levels (≥568 pmol/mL) had a strikingly different outcome from the quartile of patients with the lowest (≤103 pmol/mL) G02 levels (P = .013); median PFS was shorter (9 vs 28 months), and the long-term survival rate lower (2-year PFS of 24% vs 70%). We conclude that more rapid disease progression and lower survival rate are associated with high circulating G02 levels at diagnosis and speculate that shed neuroblastoma tumor gangliosides play a role in accelerating tumor progression.

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MATERIALS AND METHODS

Patients. Seventy-four children with histologically confirmed, advanced stage, neuroblastoma, diagnosed between 1979 and 1987, were studied. Thirty-two patients had stage III, 42 had stage IV, and none had stage IVs disease according to the Children's Cancer Study Group (CCSG) classification.1 The median patient age at the time of diagnosis was 29 months (range, 1 to 128 months). All patients received similar intensive chemotherapy at CCSG member institutions on CCSG protocols, and some also underwent surgical resection, radiation therapy, and/or bone marrow transplantation.

Patient samples. Plasma/serum specimens were obtained at the time of diagnosis, before the institution of therapy, and were stored at −70°C until the time of ganglioside analysis.

Ganglioside purification and quantitation. Gangliosides were purified from plasma or serum by a recently developed micro-method that consists of total lipid extraction of the sample with chloroform/methanol, partition of the total lipid extract in diisopropl ether/1-butanol/water, and gel filtration.2,4 The purified total circulating gangliosides were analyzed by high-performance thin-layer chromatography (HPTLC), using HPTLC plates (Merck, Darmstadt, FRG) developed in chloroform-methanol-0.25% aqueous CaCl2 (60:40:9) and stained with resorcinol. The G02 in patient samples was quantitated by densitometric scanning of the HPTLC band.

Clinical evaluation. The patient's medical record was reviewed to determine the initial progression-free period. Physical examination, isotopic bone scans, computerized axial tomography, magnetic resonance imaging, urinary catecholamine quantitation, and bone marrow histopathology were used to monitor patients. The endpoint of the clinical analysis was the documentation of progressive disease (PD), defined as an increase by 25% of established primary disease, the recurrence of marrow disease, or the occurrence of any new lesion.5 Absence of disease progression (NPD) (ie, stable disease, regressing disease, or disease in clinical remission) was confirmed by contact with the primary physician of each patient, most recently in March 1989.

Statistical analysis. The relationship between progression of disease and clinical stage, age at diagnosis, and circulating G02 (log G02) concentration was evaluated by the univariate proportional hazards regression model using the Cox and Oakes test.10 To quantify the relationship between circulating G02 levels at diagnosis and PFS, patients were grouped into quartiles by their G02 levels. The impact of the most disparate G02 levels was determined by comparison of patients in quartiles 1 and 4, analyzed by the chi-square test at the 2-year time-point, and by the method of Kaplan-Meier11 to estimate the PFS distribution for these groups. The log rank statistic12 was used to test equality of PFS curves, and the 95% confidence intervals (CI) were computed using the modified reflected method.12
GANGLIOSIDE SHEDDING AND TUMOR PROGRESSION

RESULTS

G\textsubscript{D2} ganglioside quantitation. The total plasma ganglioside patterns of three neuroblastoma patients are shown in Fig 1. They show the presence of tumor-derived G\textsubscript{D2}. The median and range of circulating G\textsubscript{D2} concentrations of all 74 patients are summarized in Table 1; the G\textsubscript{D2} content of some samples has been previously reported.\textsuperscript{13} The range of G\textsubscript{D2} concentrations varied from below the limit of chemical detection (<2 pmol/mL) to more than 2 \times 10^7 pmol G\textsubscript{D2}/mL plasma, or 1,000 times the normal plasma G\textsubscript{D2} concentration of <2 pmol/mL,\textsuperscript{13} and the median circulating G\textsubscript{D2} concentration of stage IV patients was not statistically different from that of stage III patients (Table 1). These results confirm our previous finding of lack of a statistically significant correlation between clinical stage and circulating G\textsubscript{D2} levels,\textsuperscript{4} and suggest that high levels at diagnosis are not merely a reflection of advanced stage. Finally, the G\textsubscript{D2} levels were unrelated to age at diagnosis (r = -.08, P = .4).

Disease status. Twenty-eight patients remain alive without PD. Two other patients died 3 and 8 months after diagnosis without PD (of complications related to therapy), and three others without PD were lost to follow-up 2, 12, and 99 months after diagnosis. The median follow-up of patients without disease progression is 18 months (range 2 to 101 months). Progressive disease occurred in the other 41 patients (55%): within 6 months of diagnosis in 25 patients, within 7 to 12 months in 7 patients, during the second year in 5 patients, and later in 4 patients. These findings yield an overall 3-year actuarial PFS of 32%, and median PFS of 15 months (CI, 8 to 28 months) for patients with advanced-stage neuroblastoma, consistent with recent survival statistics.\textsuperscript{14}

When patient outcome was analyzed according to stage, 34% of stage III patients had PD (median PFS, >101 months), in contrast to 71% of stage IV patients (median PFS, 8 months). Thus, clinical stage was significantly related to PFS (P < .001), as expected.\textsuperscript{7} A negative prognostic impact of older age at diagnosis\textsuperscript{15} was not confirmed in this study (P > .4), possibly because there were too few infants (n = 13).

Pretreatment circulating G\textsubscript{D2} concentration and progression-free survival. The relationship between pretreatment G\textsubscript{D2} levels and the occurrence of PD within 2 years of diagnosis is shown in Table 2 and Fig 2. All patients who are actually evaluable for disease progression at 2 years (ie, surviving 2 years without PD or having had PD documented within 2 years of diagnosis) were included (n = 52). Of the patients whose circulating G\textsubscript{D2} levels were in the highest (first) quartile, only 46% had PD, in contrast to 92% of the patients whose circulating G\textsubscript{D2} levels were in the lowest (fourth) quartile (P = .01). A continuous increase in the percentage of patients with PD is observed as the G\textsubscript{D2} concentration increases from the lowest to the highest quartile (Fig 2). Thus, the occurrence of PD is directly related to the G\textsubscript{D2} level at diagnosis. This is consistent with the hypothesis that shedding of tumor gangliosides may be an important factor in tumor progression.

A highly significant relationship between the pretreatment circulating G\textsubscript{D2} concentration and PFS was also demonstrated when the log G\textsubscript{D2} concentration was considered as a continuous variable. PFS decreased with increasing G\textsubscript{D2} level at diagnosis (P = .018). When patients were grouped into quartiles and those with the most disparate G\textsubscript{D2} levels compared by life-table analysis (quartiles 1 v 4, Fig 3), a striking difference in survival (P = .013) between these two groups was demonstrated; patients in the highest quartile had a median PFS of only 9 months (CI, 3 to 16 months), versus 28 months (CI, 8 to >99 months) for those in the lowest quartile. Thus, there is both an increase in the number of patients with progressive disease (ie, reduced long-term PFS, Fig 2) and an acceleration of the rate of disease progression (Fig 3), in association with high pretreatment circulating tumor ganglioside levels.

The potential clinical application of these results is demonstrated by the analysis of a group of patients previously assumed to have a uniform prognosis. This group, children

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**Table 1. Circulating G\textsubscript{D2} Ganglioside Concentration at Diagnosis in Patients With Advanced Stage Neuroblastoma**

<table>
<thead>
<tr>
<th>Clinical Stage</th>
<th>Circulating G\textsubscript{D2} Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median</td>
</tr>
<tr>
<td>III (n = 32)</td>
<td>178*</td>
</tr>
<tr>
<td>IV (n = 42)</td>
<td>295</td>
</tr>
<tr>
<td>III + IV (n = 74)</td>
<td>237</td>
</tr>
</tbody>
</table>

*pmol/mL; stage III versus IV, P > .05.

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Table 2. Outcome of Patients Two Years Postdiagnosis Related to Pretreatment Circulating GD2 Concentration

<table>
<thead>
<tr>
<th>Quartiles</th>
<th>Range (pmol/mL)</th>
<th>PD (n = 37)</th>
<th>NPD (n = 15)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1+</td>
<td>550-125</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>2</td>
<td>125-244</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>250-590</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>704-2,160</td>
<td>12</td>
<td>1</td>
</tr>
</tbody>
</table>

Abbreviations: PD, progressive disease; NPD, stable disease.

*All patients surviving 2 years without PD or having had PD within 2 years of diagnosis are included (n = 52); patients with NPD but follow-up of less than 24 months are excluded.

+Patients were grouped into quartiles (n = 13) according to the GD2 concentration: 1, lowest; 4, highest.

with stage IV disease who are older than 1 year at the time of diagnosis, have an almost uniformly fatal outcome (<20% long-term survival). However, the 36 such patients in this study showed marked heterogeneity of circulating GD2 levels at diagnosis. Fifteen of the 36 patients had circulating GD2 levels in the highest quartile. Fourteen of these 15 patients (94%) had PD by 1 year from diagnosis. In contrast, only 11 of 21 patients (53%) with lower GD2 concentrations had PD at this time. Thus, despite otherwise poor prognostic factors (age and stage), GD2 levels below those of the highest quartile (ie, ≤568 pmol/mL) were associated with an almost 50% chance for survival, compared with the much lower survival predicted for this group on the basis of age and stage alone.

DISCUSSION

This study shows the existence of a relationship between circulating tumor-derived ganglioside concentration at diag-

nosis and subsequent tumor progression in patients with advanced (high-risk) neuroblastoma. High circulating levels of the tumor-derived ganglioside, GD2, were strongly related to more rapid disease progression and lower survival rates. This conclusion in humans is consistent with the results of previous work probing a role of shed gangliosides in the process of tumor progression. Tumor gangliosides are shed in substantial quantities into the tumor cell microenvironment in vitro, and in vivo in a number of different tumor systems, and there is direct evidence in experimental animals that they enhance tumor formation. Observations consistent with those cited above have also been made in human neuroblastoma. Specifically, neuroblastoma tumor gangliosides, of which GD2 is the most extensively studied, are expressed in substantial quantities by tumor tissue, and GD2 is shed into the circulation of patients with neuroblastoma. Also, in a small number of patients we have studied sequentially, circulating GD2 decreased in response to therapy and increased with disease recurrence. The present findings associating accelerated tumor progression with high pretreatment circulating tumor ganglioside levels further support the view that tumor gangliosides may be a modulating factor in tumor-host interactions.

The mechanism by which shed gangliosides influence tumor growth remains to be established. Because tumor gangliosides are immunosuppressive in vitro, one possible mechanism by which shed gangliosides could enhance tumor progression is by abrogation of host immunologic anti-tumor defenses. The finding that neuroblastoma-derived gangliosides (particular GD2) depress normal in vitro cellular immune responses lends indirect support to this hypothesis.

Molecular tumor markers have previously been proposed to have prognostic significance in neuroblastoma. However, to our knowledge, tumor-derived gangliosides are the first circulating marker for which a pathogenetic role in tumor
progression has been proposed (in contrast to ferritin, neuron-specific enolase, and catecholamines). The other marker for which a pathogenetic role has been suggested is the N-myc oncogene; amplification of the N-myc oncogene is believed to impart a high proliferative capability to the tumor cell. These two characteristics may be complementary: a tumor cell phenotype exhibiting both amplification of N-myc and significant ganglioside shedding may result in a tumor that is particularly aggressive. This hypothesis and possible correlations between circulating G0₂ and other biologic markers of neuroblastoma warrant testing.

In conclusion, the present data relate significant shedding, measured as high levels of circulating tumor-derived gangliosides at diagnosis, to acceleration of tumor progression in humans, and suggest the consideration and further study of these molecules as circulating tumor markers of biologic significance.

ACKNOWLEDGMENT

We are grateful to the CCSG Neuroblastoma Reference Laboratory and Dr Robert Seeger for cooperation in this study and for providing certain patient samples; and we thank Shirley Brown for preparation of the manuscript.

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Shed tumor gangliosides and progression of human neuroblastoma

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