Quantitative Tumor Cell Content of Bone Marrow and Blood as a Predictor of Outcome in Stage IV Neuroblastoma: A Children's Cancer Group Study

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Purpose: **This study investigated the prognostic value of quantifying tumor cells in bone marrow and blood by immunocytology in children with high-risk, metastatic neuroblastoma.**

Patients and Methods: **Patients with stage IV neuroblastoma (N = 466) registered on Children's Cancer Group study 3891 received five cycles of induction chemotherapy and were randomized either to myeloablative chemoradiotherapy with autologous purged bone marrow rescue or to nonmyeloablative chemotherapy. Subsequently, they were randomized to 13-*cis*-retinoic acid or no further treatment. Immunocytologic analyses of bone marrow and blood were performed at diagnosis, week 4, week 12, bone marrow collection, and end induction and were correlated with tumor biology, clinical variables, treatment regimen, and event-free survival (EFS).**

Results: **Immunocytology identified neuroblastoma cells in bone marrow of 81% at diagnosis, 55% at 4 weeks, 27% at 12 weeks, 19% at bone marrow collection, and 14% at end induction. Tumor cells were detected in blood of 58% at diagnosis and 5% at collection. There was an adverse effect on EFS of increasing tumor cell concentration in bone marrow at diagnosis ($P = .04$), at 12 weeks ($P = .006$), at bone marrow collection ($P < .001$), and at end induction ($P = .07$). Positive blood immunocytology at diagnosis was associated with decreased EFS ($P = .003$). The prognostic impact of immunocytology was independent of morphologically detected bone marrow disease, *MYCN* status, and serum ferritin level in bivariate Cox analyses.**

Conclusion: **Immunocytologic quantification of neuroblastoma cells in bone marrow and blood at diagnosis and in bone marrow during induction chemotherapy provides prognostic information that can identify patients with very high-risk disease who should be considered for experimental therapy that might improve outcome.**

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BONE MARROW involvement by neuroblastoma is extremely common in children with metastatic disease, present by standard morphologic examination of aspirates and biopsies in 60% to 65% of children with stage IV disease at diagnosis.[1] Detection of tumor by immunocytology, using a mixture of monoclonal antibodies reactive at the cell surface, has been shown to reliably detect tumor with a sensitivity that may vary from 1 to 10 in 10^5 nucleated bone marrow cells, depending on the method of detection.[2-14] Detection of small numbers of contaminating tumor cells in bone marrow or blood may be a very important component of response evaluation and is critical for evaluation of hematopoietic stem cell products, because the use of myeloablative therapy followed by autologous bone marrow transplantation now has been shown in a randomized trial to be beneficial to event-free survival (EFS).[15]

The goal of this study was to determine whether quantitative sequential assessment of bone marrow and peripheral-blood tumor cells was prognostic for disease response at the end of induction therapy and for ultimate EFS. We report here the correlation of bone marrow and blood immunocytology with outcome for all assessable patients with metastatic neuroblastoma treated on Children's Cancer Group (CCG) protocol CCG-3891.[15] This protocol was a phase III study using a standard induction chemotherapy followed by randomization to myeloablative chemoradiotherapy with purged autologous bone marrow transplantation (ABMT) or to intensive nonmyeloablative consolidation chemotherapy, and a second randomization to no further treatment or 6 months of 13-*cis*-retinoic acid biotherapy.

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PATIENTS AND METHODS

Patients

Eligible patients included newly diagnosed children (1 to 18 years of age) enrolled on the CCG-3891 protocol for high-risk neuroblastoma. Protocol eligibility included patients with the following clinical and biologic characteristics: age ≥ 1 year and Evans stage IV disease (n = 434); age less than 1 year with stage IV and tumor *MYCN* gene amplification (n = 19). Thirteen additional children who had localized disease treated with surgery alone at diagnosis but who then developed metastases and enrolled on CCG-3891 were also included. Other high-risk patients enrolled on the CCG-3891 study but who lacked metastases were excluded from the analyses reported in this article in order to examine only children with stage IV disease. Signed informed consent by parents or guardians was obtained, along with appropriate local institutional review board approval. Patient accrual began January 1991 and ended April 1996.

Serum ferritin was measured by radioimmunoassay: levels ≥ 143 ng/mL were designated as unfavorable and levels less than 143 ng/mL were designated as favorable.[16] Biologic features of tumor, including histology and *MYCN* gene copy number, were determined centrally in the CCG Neuroblastoma Biology Resource Laboratory. Tumor *MYCN* gene amplification was measured by Southern blot analysis (1991 through 1993) as previously described[17] by both the pattern and intensity of *MYCN* protein expression by immunohistochemistry and by semiquantitative polymerase chain reaction (1994 through 1996).[18] Central pathology review of all tumors was performed, with classification as favorable or unfavorable according to the Shimada classification system.[19]

Treatment

All patients received initial therapy with cisplatin 60 mg/m^2 on day 0, doxorubicin 30 mg/m^2 on day 2, etoposide 100 mg/m^2 on days 2 and 5, and cyclophosphamide 1,000 mg/m^2 on days 3 and 4 for five cycles at 28-day intervals, plus surgery and radiotherapy for gross residual disease. For patients randomized to ABMT, pretransplantation therapy consisted of continuous-infusion carboplatin 1,000 mg/m^2/96 hours on days −8 to −5 and etoposide 640 mg/m^2/96 hours on days −8 to −5, bolus melphalan 140 mg/m^2 on day −7 and 70 mg/m^2 on day −6, and total-body irradiation (3.33 Gy/d on days −3 to −1), followed by purged ABMT (median 2 × 10^8 mononuclear cells/kg) on day 0, followed by granulocyte colony-stimulating factor. The continuation chemotherapy arm used three cycles of continuous-infusion cisplatin 160 mg/m^2/96 hours, etoposide 500 mg/m^2/96 hours, and doxorubicin 40 mg/m^2/96 hours simultaneously with bolus ifosfamide 2,500 mg/m^2/d on days 0 through 3 and mesna followed by granulocyte colony-stimulating factor. The first randomization occurred just before the third cycle of initial induction therapy, at protocol week 8 (median, 59 days) for patients without disease progression. At the end of continuation therapy or ABMT (protocol week 34 from diagnosis; median, 285 days), patients without disease progression underwent a sequential second randomization to receive either six cycles of 13-*cis*-retinoic acid 160 mg/m^2/d orally in two divided doses for 14 consecutive days in a 28-day cycle or no further therapy.[15] Disease evaluations were performed at diagnosis and at the end of induction, after ABMT or continuation chemotherapy, and after retinoid therapy. Responses were assessed according to the International Neuroblastoma Response Criteria.[20]

Bone Marrow and Blood Immunocytology, Bone Marrow Collection, and Purging

Heparinized samples of peripheral blood and bilateral pooled posterior iliac crest bone marrow aspirates were sent to the CCG Neuroblastoma Resource Laboratory for analysis at the designated time points. Bone marrow was sent at diagnosis, 4 weeks (after one cycle of chemotherapy), 12 weeks (after three cycles of chemotherapy), precollection, at bone marrow collection, and at the end of the five cycles of induction chemotherapy. Peripheral blood was sent at diagnosis, week 12, and at collection. Samples were analyzed as previously described using a mixture of five monoclonal antibodies (HSAN1.2, 390, 459, 126-4, and BW575) with a sensitivity of one tumor cell per 10^5 nucleated cells.[2]

Bone marrow was collected for ABMT and processed at the CCG Neuroblastoma Purging Center just before the fourth or fifth cycle of initial therapy if a diagnostic bone marrow aspirate obtained a week before collection had less than 1% tumor by morphologic and immunocytologic analysis. Bone marrow was purged using sedimentation, filtration, and two cycles of immunomagnetic separation.[21-23] All infused marrows were tumor-free by bone marrow immunocytology.

Statistical Design and Analysis

Life-table methods were used to estimate EFS.[24] An event was defined as the first occurrence of any disease progression, second malignancy, or death from any cause. EFS was calculated from various time points corresponding to the times of sample acquisition. Thus EFS from time of study entry was considered when investigating the prognostic power of blood or bone marrow immunocytology from samples taken at entry, whereas EFS from time of collection was considered when evaluating the prognostic power of immunocytology of samples taken at collection. The log-rank statistic was used to compare the EFS probabilities between subgroups of patients, with subgroups defined according to level of blood or bone marrow tumor content. When more than two levels were considered, tests for trend in hazard were constructed using the approach of Thomas et al.[25] Relative-risk analysis was performed using the regression method of Cox.[26] Multivariate Cox models were used to assess the independence of predictive information coming from blood and bone marrow immunocytology relative to other known prognostic factors, including serum ferritin, *MYCN* amplification, and Shimada histopathology classification.[15]

RESULTS

Patients and Bone Marrow Immunocytology Samples

A total of 466 children with stage IV or previously untreated low-stage disease that had metastasized were enrolled on CCG-3891. Of these patients, 422 had at least one bone marrow sample available for immunocytologic analysis. A total of 2,626 bone marrow immunocytology samples were obtained from these patients from diagnosis through the end of consolidation therapy. Only those obtained at diagnosis (n = 267), 4 weeks from diagnosis (n = 262), 12 weeks from diagnosis (after three cycles of induction chemotherapy; n = 244), 1 week before collection (n = 171), from the collected bone marrow (n = 200), and at the end of induction, approximately 20 to 24 weeks from

Table 1. Bone Marrow and Blood Immunocytology*

	Week 0	Week 4	Week 12	At Bone Marrow Collection	End Induction
Bone Marrow	n = 267	n = 262	n = 244	n = 200	n = 138
Patients with 0,† n	51	117	178	163	119
Patients with 1-99, n	31	86	51	30	16
Patients with ≥ 100, n	185	59	15	7	3
Samples > 0					
Median‡	8674	60	15	5	3
Range	2-99,000	1-80,000	1-69,444	1-5117	1-257
Blood	n = 174	n = 23	n = 98	n = 158	n = 18
Patients with 0,† n	73	22	93	150	18
Patients with 1-99, n	68	1	5	8	0
Patients with ≥ 100, n	33	0	0	0	0
Samples > 0					
Median‡	28	—	1	3	—
Range	1-19,859	—	1-68	1-20	—

*Analysis was performed on bone marrow or blood prepared by density separation using Histopaque-1077 (Sigma Chemical Co, St Louis, MO) and centrifugation.

†Values shown are for the number of patients in each group having either 0, 1-99, or ≥ 100 tumor cells per 10^5 nucleated cells.

‡The median cell number expressed as tumor cells per 10^5 nucleated cells.

diagnosis (n = 138), were included in the analyses here. Twenty-one patients had samples at six time-points, 62 patients had samples at five time-points, 92 patients had samples at four time-points, 80 patients had samples at three time-points, 83 patients had samples at two time points, and 72 patients had only a single sample available. The precollection sample was not examined separately for EFS and other associations, because it was obtained in such close proximity to the bone marrow collection. Samples for blood immunocytology were also obtained at diagnosis (n = 174), 4 weeks (n = 23), 12 weeks (n = 98), and at the time of bone marrow collection (n = 158).

The quantitation of tumor by immunocytology at the various time points used for analysis is shown for bone marrow and blood in Table 1 and Fig 1. Patient characteristics and the percentage of patients with positive samples by characteristic at the various time points for both blood and bone marrow immunocytology are listed in Table 2.

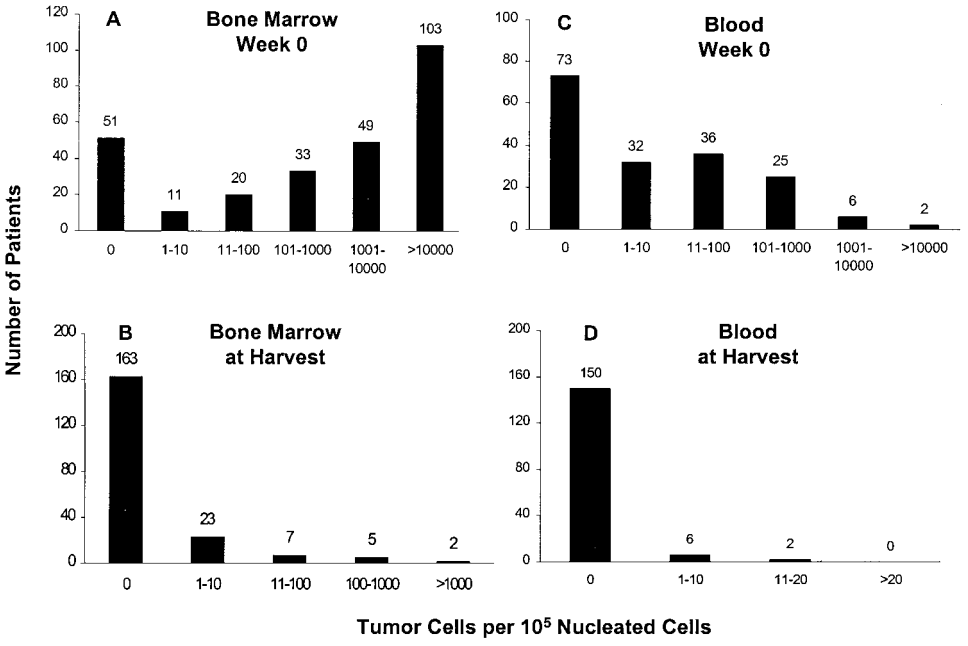

Fig 1. Quantification of neuroblastoma cells in bone marrow and peripheral blood by immunocytology at diagnosis and at bone marrow collection. Bone marrow at (A) diagnosis and (B) at collection; peripheral blood at (C) diagnosis and at (D) collection.

Table 2. Patient Characteristics and Correlation With Immunocytologic Detection of Tumor Cells in Blood and Bone Marrow

		Patients With Positive Immunocytology (%)						
	All Stage IV	Bld-Week 0 n = 174	Bld-Collection n = 158	BM-Week 0 n = 267	BM-Week 4 n = 262	BM-Week 12 n = 244	BM-Collection n = 200	BM-End Induction n = 138
All Stage IV	466	58	5	81	55	27	19	14
MYCN								
Amplified	131	71*	8	81	49	17	20	6
Nonamplified	202	48	5	82	56	29	17	17
Shimada								
Unfavorable	299	53	5	80	50	23	17	11
Favorable	15	57	0	70	57	38	17	0
Ferritin								
≥ 143 ng/mL	249	64*	7	83	64*	33*	21	15
< 143 ng/mL	167	44	4	77	41	15	14	12
Bone metastases								
Yes	318	60	6	86*	63*	29	24*	17
No	148	55	2	71	40	22	5	5
Morphologic BM metastases*								
Yes	374	63*	5	92*	61*	31*	21	15
No	92	26	3	38	19	11	9	0
End induction								
CR/VGPR	112	65	2	87	47*	33	13	11
PR	84	47	0	82	63	24	21	19
Randomized								
ABMT	168	57	5	83	59	26	22	14
CC	166	54	5	80	57	28	13	11
Randomized								
No 13-*cis*-RA	111	67	2	83	59	30	7	15
13-*cis*-RA	107	57	5	74	52	19	16	12

Abbreviations: Bld, blood; BM, bone marrow; *MYCN* amplified, tumor with ≥ 10 gene copies; *MYCN* nonamplified, < 10 copies; BM metastases, bone marrow metastases by morphologic examination of bilateral aspirates and biopsies; positive immunocytology, ≥ 1 tumor cell per 10^5 nucleated cells; CC, consolidation chemotherapy; 13-*cis*-RA, 13-*cis*-retinoic acid; CR, complete remission; VGPR, very good partial remission; PR, partial remission, by international response criteria.[20]

*Significant difference between the two groups, $P < .05$, for the observed/expected ratio.

The median age for these patients was 2.9 years, with a typical profile of clinical and biologic risk factors for children with stage IV neuroblastoma ≥ 1 year of age. Thirty-nine percent of patients had *MYCN*-amplified tumors, 60% had elevated serum ferritin, 95% had unfavorable histology, 68% had bone metastases, and 80% had bone marrow metastases (by morphologic examination of aspirates and biopsies).

Eighty-one percent of children had a positive bone marrow immunocytology at diagnosis, with a median bone marrow tumor burden for positive samples of 8,674 cells per 10^5 nucleated cells (Table 1). The concordance of light microscopic evidence of bone marrow metastases by the local institutional pathologist (which included bilateral aspirate and biopsy) with bone marrow immunocytology was 86%. Thirty-three bone marrow samples were negative by both evaluations, 196 were positive by both evaluations, and 38 (14%) were discordant. Eighteen marrow samples were positive by light microscopic evaluation and negative by immunocytology, whereas 20 were negative by light microscopic evaluation and positive by immunocytology. When the precollection and collection samples were compared using a quantitative cutoff of fewer than 100 versus ≥ 100 tumor cells per 10^5 bone marrow cells, they were 96% concordant in 171 patients. However, there was lower concordance (71%) when considering whether samples had only one or more tumor cell per 10^5 bone marrow cells, probably because of the much larger volume of the sample (100-fold) obtained at actual collection compared with that obtained at precollection.

The percentage of patients with any detectable bone marrow tumor by immunocytology steadily decreased during therapy, as shown in Fig 1, Table 1, and Table 2, from 81% of patients at diagnosis to 14% by the end of induction. If the cutoff of ≥ 100 tumor cells per 10^5 nucleated cells is used for the time points of diagnosis, 4 weeks, 12 weeks, collection, and end of induction therapy, the percentages positive were 69%, 23%, 6%, 4%, and 2%, respectively. In contrast to the results in bone marrow, no further decrement in circulating tumor was seen after 4 weeks (Table 1). Blood

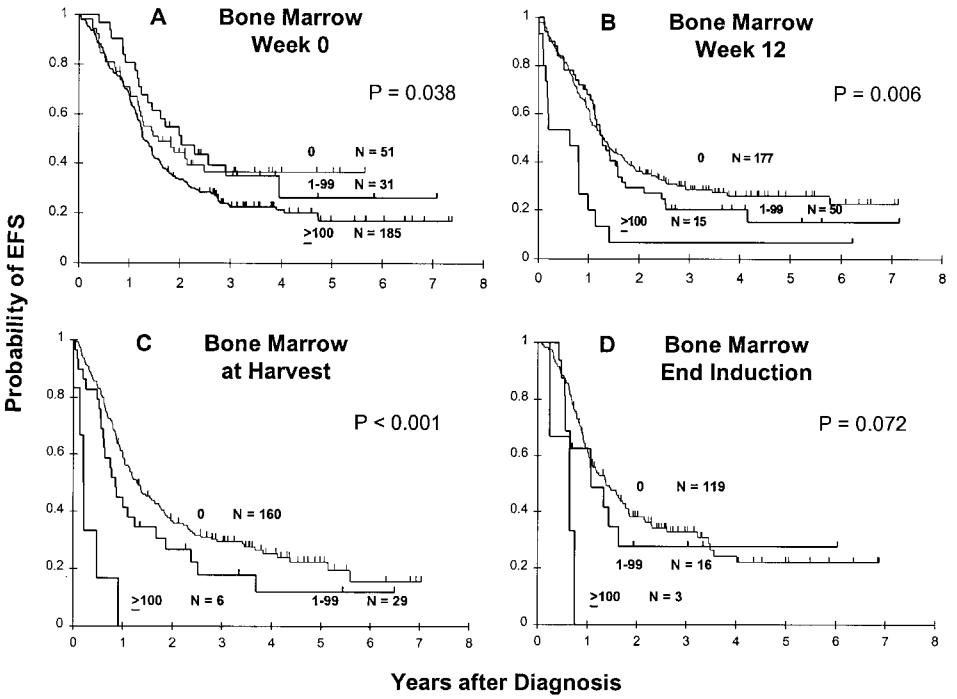

Fig 2. Neuroblastoma cells in bone marrow and EFS. Immunocytology was performed at (A) week 0 (diagnosis), (B) week 12, (C) the time of marrow collection, and (D) the end of induction chemotherapy. Patient groups with 0, 1 to 99, and ≥ 100 tumor cells per 10^5 nucleated bone marrow cells and the number of patients in each group are indicated.

samples for immunocytology at diagnosis, week 4, week 12, and collection showed circulating tumor cells (≥ one tumor cell per 10^5) at these times in 58%, 4%, 5%, and 5% of patients, respectively.

Children with high-risk stage III neuroblastoma were also entered on CCG-3891 (n = 72). Their results are not included in the analyses that follow in this article in order to have a uniform group of stage IV patients, but the immunocytology quantitation is given here for comparison. Immunocytology at diagnosis was positive in nine of 36 children with stage III disease, with a median tumor cell number of 7 per 10^5 nucleated cells, and blood immunocytology was positive in only two of 19 patients. Bone marrow immunocytology at 4 weeks was positive in two of 18, at 12 weeks in one of 27, at collection in 0 of 18, and at end induction in 0 of 23 patients. There was no significant difference in EFS by bone marrow immunocytology at diagnosis. Only one patient with stage III neuroblastoma had positive blood immunocytology at both 12 weeks and harvest, but not at the end of induction. This patient experienced relapse and died.

Immunocytology and Biologic and Clinical Risk Factors

Association of immunocytology with prognostic factors, sites of metastases and response to therapy were evaluated using the χ^2 test of proportions (Table 2). Positive immunocytology in bone marrow at diagnosis was associated with both bone and morphologic bone marrow metastases, whereas positive immunocytology in blood at diagnosis was associated significantly only with morphologic bone marrow metastases. Positive blood immunocytology at diagnosis also was associated with the unfavorable biologic factors of elevated serum ferritin and tumor *MYCN* gene amplification. Negative bone marrow immunocytology at week 4 was associated with complete or very good partial response at the end of induction, whereas positive immunocytology at week 4 showed a trend ($P = .065$) toward association with partial response.

Immunocytology and Prognosis

The relationship of EFS to quantitative bone marrow immunocytology at time of diagnosis, 12 weeks, the time of collection, and end of induction just before consolidation therapy is shown in Fig 2. Results were similar for disease-free survival, with a slight decrease in significance ($P = .069$) for immunocytology at diagnosis. There was an adverse effect of increasing tumor cell concentration in bone marrow on EFS both at diagnosis ($P = .038$), 12 weeks ($P = .006$), and collection ($P < .001$). There was also a trend at the end of induction therapy ($P = .072$), whether the patient went on to ABMT or consolidation chemotherapy. The result of immunocytology at 4 weeks, after only one cycle of chemotherapy, was not associated with EFS ($P = .468$), even though it was correlated with

Table 3. EFS According to Quantitative Bone Marrow Immunocytology and Treatment Arm

	All Patients		CC		ABMT		13-*cis*-RA		No 13-*cis*-RA	
	0 *v* ≥ 1	0-99 *v* ≥ 100	0 *v* ≥ 1	0-99 *v* ≥ 100	0 *v* ≥ 1	0-99 *v* ≥ 100	0 *v* ≥ 1	0-99 *v* ≥ 100	0 *v* ≥ 1	0-99 *v* ≥ 100
BM-0										
3-year EFS, %	37 *v* 24	36 *v* 22	19 *v* 21	25 *v* 18	55 *v* 26	42 *v* 26	50 *v* 41	45 *v* 42	41 *v* 21	32 *v* 20
P	NS	.018	NS	NS	NS	NS	NS	NS	NS	NS
BM-4										
3-year EFS, %	31 *v* 27	30 *v* 25	26 *v* 22	22 *v* 29	34 *v* 29	33 *v* 24	45 *v* 41	46 *v* 33	30 *v* 31	28 *v* 37
P	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
BM-12										
3-year EFS, %	28 *v* 17	27 *v* 7	24 *v* 11	22 *v* 0	29 *v* 21	27 *v* 13	42 *v* 34	41 *v* NA	19 *v* 32	23 *v* 25
P	.06	.0002	NS	.002	NS	.054	NS		NS	NS
BM-at collection										
3-year EFS, %	30 *v* 15	28 *v* 0	25 *v* 0	23 *v* 0	29 *v* 19	27 *v* 0	38 *v* 44	39 *v* NA	27 *v* *	26 *v* NA
P	.009	< .0001	.071	.057	.069	< .0001	NS		.037	
BM-end induction										
3-year EFS, %	33 *v* 23	32 *v* 0%	28 *v* 30	29 *v* 0	36 *v* 30	36 *v* NA	50 *v* 50	49 *v* NA	26 *v* 14	24 *v* NA
P	NS	.001	NS	< .001	NS		NS		NS	

Abbreviations: NA, not applicable, no samples with ≥ 100 tumor cells; NS, not significant.

*One of four patients alive and disease-free at 1 year.

induction response ($P = .0178$) (Table 2). A significant difference, listed in Table 3, was also seen in EFS at weeks 0 and 12, collection, and preconsolidation using a cutoff point of ≥ 100 tumor cells versus fewer than 100 tumor cells per 10^5 nucleated cells, with 3-year EFS at diagnosis for that level of tumor cells of 36% versus 22% ($P = .018$), 27% versus 7% at 12 weeks ($P = .0002$), 28% versus 0% at the time of collection ($P < .0001$), and 32% versus 0% at the end of induction therapy ($P = .0014$). Bone marrow immunocytology was also a highly significant ($P < .0001$) adverse factor using a cutoff tumor cell quantity of more than 20 tumor cells per 10^5 nucleated cells at the time of bone marrow collection, although only four patients had between 20 and 100 tumor cells at this time.

To test whether the patients with missing samples differed from those with immunocytology results, an analysis of EFS in these two subgroups was performed. There was no significant difference in EFS for patients with metastatic disease lacking immunocytology and those with this result available. Three-year EFS for those lacking an immunocytology sample at diagnosis compared with those with an immunocytology result using the log-rank test was 26% versus 27% ($P = .21$), 27% versus 25% at week 12 ($P = .65$), and 28% versus 32% at the end of induction ($P = .19$).

This adverse prognostic impact of bone marrow immunocytology ≥ 100 tumor cells per 10^5 nucleated cells at weeks 0, 12, or at collection remained significant ($P < .05$) in bivariate Cox analyses that included *MYCN* amplification or serum ferritin. Including Shimada classification as a predictor reduced the significance of the week 0 immunocytology in the analysis ($P < .3$), but this may be partly explained by reduced patient numbers, because 84 patients with immunocytology at diagnosis were missing the Shimada classification. After stratification by morphologic evidence of neuroblastoma in bone marrow, patients with ≥ 100 tumor cells in marrow at diagnosis had a significantly lower 3-year EFS (22%) than those with fewer than 100 tumor cells (44%; $P = .016$). However, for those patients with morphologically negative bone marrows at diagnosis (n = 53), the detection of ≥ 100 tumor cells by immunocytology was not predictive ($P = .65$). Nor was a difference seen ($P = .8$) when the patients who were negative by both measures were compared with those who were positive by light microscopy but negative by immunocytology (n = 20). At the end of induction, there were only five patients who were negative by light microscopy but positive on immunocytology, of whom three experienced relapse. Multivariate analysis of 139 patients with all four variables available, including immunocytology (≥ 100 tumor cells at diagnosis), *MYCN*, ferritin, and Shimada classification, showed the same increased relative risk for immunocytology of 1.5 (95% confidence interval, 0.93 to 2.29) as in univariate analysis, but only *MYCN* retained a significant *P* value.

Positive blood immunocytology at week 0 but not at collection (Fig 3) was correlated with decreased EFS. In contrast to the bone marrow results, the blood immunocytology at week 0 was no longer predictive ($P > .9$) in a model that included *MYCN* amplification.

When analyzed by treatment regimen, there was still an EFS advantage for patients with fewer than 100 tumor cells at diagnosis, 12 weeks, and at bone marrow collection, regardless of whether they were treated with ABMT or

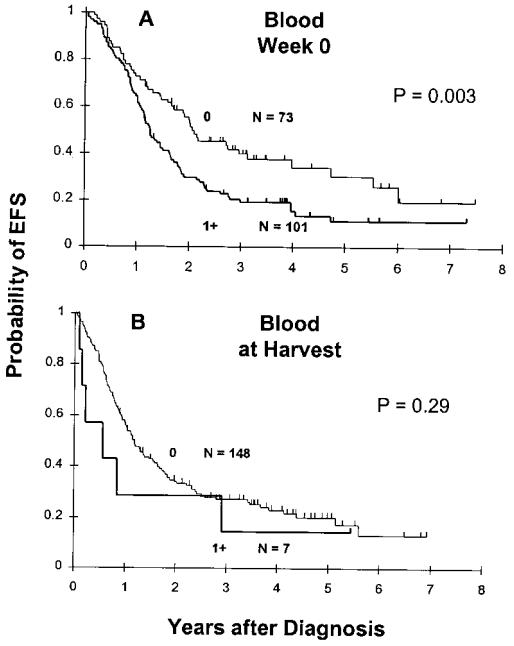

Fig 3. Neuroblastoma cells in peripheral blood and EFS. Immunocytology was performed at (A) diagnosis and (B) at the time of marrow collection. Patient groups with 0 or ≥ 1 (1+) tumor cells per 10^5 nucleated blood cells and the number of patients in each group are indicated.

consolidation chemotherapy (Table 3). However, this was only significant ($P < .05$) in the 12-week (chemotherapy arm) or collection (ABMT arm) samples. There was not a significant difference in EFS for the subgroups of patients with either positive (≥ one tumor cell per 10^5) or negative immunocytology at any of the time points by consolidation regimen. The only difference for immunocytology after stratification by postconsolidation therapy (± 13-*cis*-retinoic acid) was for patients who had positive immunocytology at collection and were randomized to no postconsolidation therapy. The four patients with positive immunocytology (≥ one tumor cell) had a significantly lower EFS than those 52 with negative immunocytology ($P = .037$). After stratification by immunocytology, a relative though nonsignificant advantage in EFS was seen for those receiving 13-*cis*-retinoic acid, consistent with the overall benefit for this arm of the study.[15]

Next, in an attempt to discover whether the amount of change in immunocytology was significant, we examined EFS in patients whose paired bone marrow samples were grouped by changes from positive to negative compared with those who did not become negative. This analysis was limited by the numbers of patients who had paired samples at the various time points. For patients with any positive bone marrow immunocytology at diagnosis, regardless of the percentage, the EFS was not significantly different whether the tumor cells had cleared at 4 weeks or at 12 weeks. There was also no significant difference in the EFS for those patients whose immunocytology at 4 weeks was positive but at 12 weeks changed to negative, regardless of whether the cutoff of one tumor cell or 100 tumor cells was considered positive. We also examined the degree of decrease in marrow tumor cells by looking at groups who cleared 1, 2, or ≥ 3 logs of tumor cells between weeks 0 and 4 or weeks 0 and 12. No significant difference was seen on the basis of the degree of decrease in bone marrow tumor cells. This is in contrast to the fact that positive immunocytology at 12 weeks was a highly significant adverse prognostic factor when analyzed alone and may reflect the limited number of paired samples submitted for immunocytology.

DISCUSSION

Our data show that quantitative bone marrow immunocytology at diagnosis is a powerful prognostic factor in children with stage IV neuroblastoma. Moreover, this factor remains influential even after consideration of other known predictors of outcome, namely *MYCN* gene copy number,[17] serum ferritin,[16] and morphologic evidence of bone marrow involvement.[1] This confirms and quantifies our previous observation that bone marrow is one of the unfavorable sites of metastases in stage IV neuroblastoma.[1] We further have shown that patients with more than 100 tumor cells per 10^5 nucleated bone marrow cells after three to four cycles of chemotherapy had virtually no chance of survival, even with the myeloablative chemoradiotherapy and purged autologous bone marrow transplantation used in this study. Patients with a small number of persisting bone marrow tumor cells at the time of collection (< 100 per 10^5 nucleated bone marrow cells) did not have significantly different EFS than those with no detectable tumor. This suggests that the intensive pretransplantation conditioning or the consolidation therapy may be sufficient to eliminate small numbers of tumor cells, but not larger amounts. The fact that one half of the patients also received 13-*cis*-retinoic acid after consolidation may also have helped to eradicate small amounts of residual bone marrow tumor cells.[15,27] However, the very small number of patients with detectable tumor cells in the bone marrow after consolidation or ABMT precluded the likelihood of seeing an impact of retinoid therapy on such patients.

It is also possible that the reason patients with small numbers of bone marrow tumor cells did not have a different EFS than those without immunocytologically detectable tumor was due to the influence of sampling variability on tumor detection. This sampling dependence was suggested by the lower concordance of immediate precol-

lection and collection immunocytology results in patients with small amounts of tumor cells. Thus there may have been some patients in the so-called negative group who had small pockets of microscopic tumor.

Although the lack of immunocytology specimens for some patients at any given time point may have lessened the power of the analyses, the overall percentage of patients with metastatic disease on CCG-3891 who had a positive bone marrow at diagnosis by immunocytology (216 of 267 or 81%) or by morphology (374 of 466 or 80%) was similar, suggesting that there was nothing different about the group of patients for whom immunocytology results were not obtained. Furthermore, an analysis of EFS in patients with metastatic disease lacking immunocytology and those with this result available showed no significant difference at diagnosis ($P = .21$), week 12 ($P = .65$), or the end of induction ($P = .19$).

The rapidity of the bone marrow response did not seem to be prognostic, as evidenced by a failure to correlate EFS with bone marrow immunocytology at week 4 after a single cycle of combination chemotherapy. Neither the conversion from positive to negative nor the extent of the change seemed to influence prognosis. This is different from the results reported for childhood acute lymphoblastic leukemia, where a day 14 and even a day 7 induction phase bone marrow are prognostic.[28]

Our data clearly demonstrate in a very large population of patients with metastatic neuroblastoma that circulating tumor cells in peripheral blood at diagnosis are extremely frequent, occurring in 58% of children. Eight patients had more than 1% tumor cells among their total nucleated cells circulating in blood. This study supports the preliminary observations of others demonstrating circulating tumor cells in the blood or in peripheral-blood stem-cell collections of some patients with neuroblastoma, using immunologic detection, clonogenic assays, and reverse-transcriptase polymerase chain reactions (RT-PCR).[29-35] Our data show that detection of circulating tumor cells in peripheral blood at diagnosis is a significant unfavorable prognostic factor and, furthermore, is correlated with other high-risk features, including *MYCN* amplification and elevated serum ferritin.

The data also show, not surprisingly, that circulating tumor cells are more likely to be found in patients with bone marrow metastases. Such peripheral-blood tumor cells persisted in a small percentage of patients even after multiple courses of chemotherapy, with little decrease subsequent to the first cycle of chemotherapy. This suggests that peripheral-blood stem-cell collections could be performed early in the course of treatment without increased risk of tumor contamination. However, the persistence of tumor cells in some patients emphasizes the importance of testing peripheral-blood stem-cell collections for tumor by sensitive methods before reinfusion and raises the question of the need for specific tumor-cell purging of stem-cell products. RT-PCR may increase the sensitivity of testing and provide an additional way to monitor circulating tumor cells.[29,33-35] Parallel testing of RT-PCR, which may be an overly sensitive method, will be necessary together with immunocytology to determine which method is more valid for prediction of outcome.

In conclusion, quantifying tumor cells in peripheral blood and bone marrow at diagnosis and during induction therapy is an important prognostic factor for patients with stage IV neuroblastoma. Moreover, bone marrow immunocytology seems to provide independent prognostic information for this group of patients, beyond other known predictors such as *MYCN* copy number and serum ferritin. The frequency of circulating tumor cells also suggests the possible need for tumor purging when peripheral-blood stem cells are used for hematopoietic support. Failure to reduce tumor cells in bone marrow to fewer than 100 tumor cells per 10^5 bone marrow cells within 12 weeks of beginning treatment is a very unfavorable prognostic sign using current therapeutic approaches and may provide a surrogate marker to detect patients who should be changed from standard therapy to an alternative, innovative treatment with greater potential to improve outcome.

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APPENDIX

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