Detecting CD56+/NB84+/CD45– Immunophenotype in the Bone Marrow of Patients with Metastatic Neuroblastoma Using Flow Cytometry

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Abstract. *Background: In order to identify neuroblastoma cells infiltrating the bone marrow, a triple-color flow-cytometric assay was developed combining CD56 and CD45 with the intracellular anti-NB84 specific antibody. Materials and Methods: The bilateral aspirates obtained from 27 consecutive children over the age of one year with stage 4 neuroblastoma were evaluated. Results: Neuroblastoma cells were detected in the bone marrow of 17/27 (63%) and 19/27 (70%) cases using cytomorphology and triple-color flow-cytometry, respectively. Using cytometry, the percentage of CD56+/NB84+/CD45– cells infiltrating the bone marrow ranged from 0.02% to 65%. Five out of eight patients without bone marrow involvement according to cytometry are in continuous complete remission, while only 3 out of 19 patients whose bone marrow gave positive results are still alive. Conclusion: By combining CD45 and CD56 with the specific antibody, NB84, directed against neuroblastoma cells, we developed a rapid and reliable cytometric assay that can be associated with conventional cytomorphological bone marrow evaluation to detect infiltrating neuroblastoma cells, especially in cases of dubious positivity.*

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Neuroblastomas (NB) are the most frequent extra-cranial solid neoplasms in young children; approximately half of the patients affected have bone marrow (BM) and/or skeletal metastases at diagnosis (1). The unambiguous identification of NB cells in the BM is, therefore, a central problem for the initial staging of these patients; reliable methods for detecting NB cells, such as flow cytometry (FC), would be helpful, but are still rarely used. NB cells are characterized by an intense expression of the 140 kDa neural cell adhesion molecule, N-CAM (CD56) (2), and by the absence of any isoform of the common leukocyte antigens recognized by the CD45 (3). The CD56+/CD45– phenotype is not restricted to NB cells; it is also displayed by small cell carcinoma (4), rhabdomyosarcoma (2), Ewing's sarcoma (5), other neuroendocrine tumors (6) and abnormal plasma cells (7). As further NB markers, CD9 (8) and CD81 (9) have been proposed in combination with CD56 and CD45. In the present study, in an attempt to develop a useful FC method for identifying BM-NB cells, anti-NB NB84 (10) was used in a triple-color FC assay with CD56 and CD45. NB84 recognizes an uncharacterized molecule of 57 kDa and its staining pattern among blue round cells tumors is restricted to NB and focal staining in PNET/Ewing sarcoma. No NB84 immunoreactivity was observed on rhabdomyosarcoma or lymphoma/leukemia (10).

Materials and Methods

Patients. During the study period (1995-2000), 27 consecutive patients over the age of one year with stage 4 NB at onset were admitted to the Istituto Nazionale per lo Studio e la Cura dei Tumori (INT) in Milan, Italy. In all cases, diagnosis and staging were performed in accordance with the International Neuroblastoma Staging System. Their median age was 44 months (13-199; Table I) and the male/female ratio was 15/12. MYCN amplifications were done in 80% of the patients, but no correlation with prognosis was observed.

Table I. *Clinical characteristics of 27 neuroblastoma patients.*

	Primary site	BM* morphology	FC BM** CD56+/NB84+/ CD45– %	Survival (months)	Status
1	R	-	-	99	RC
2	R	-	-	80	RC
3	R	-	-	76	AWD
4	M	-	-	74	RC
5	A	-	-	41	DOD
6	M	-	-	53	RC
7	R	-	-	57	RC
8	A	-	-	10	DOD
9	A	-	0.02	25	DOD
10	A	-	0.09	29	DOD
11	M	+	0.30	44	DOD
12	M	+	0.50	96	RC
13	M	+	0.50	11	DOD
14	A	+	1.50	6	DOD
15	A	+	1.70	27	DOD
16	A	+	2.00	29	DOD
17	A	+	2.50	12	DOD
18	A	+	2.50	9	DOD
19	M	+	3.20	34	DOD
20	A	+	10.0	51	RC
21	A	+	10.0	20	DOD
22	A	+	17.0	15	DOD
23	A	+	18.0	3	DOD
24	R	+	20.0	16	DOD
25	A	+	27.0	4	DOD
26	A	+	50.0	79	RC
27	A	+	65.0	14	DOD

A: adrenal gland; M: mediastinum; R: retroperitoneum.
*The BM was scored as positive if at least 1 of 4 sites revealed neoplastic cells.
**The percentage of CD56+/NB84+/CD45– cells was calculated, as explained in the Methods; the number reported in the column was the mean of the percentage obtained from both aspirates (from left and right iliac crest).
RC: complete remission; AWD: alive with disease; DOD: died of disease.

When possible, 2 bilateral aspirates and 2 bilateral BM biopsies were obtained as part of the staging procedure (from 4 different sites, in the left and right posterior iliac crest). All patients were treated according to the INT protocol for stage 4 NB, consisting of 1 cycle every month for 6 months, intensive therapy, surgery on the primary tumor site whenever feasible, and then myeloablative chemotherapy plus autologous hemopoietic stem cell rescue. The parents' informed consent was obtained in all cases for the study and the treatment plan. Five additional surgical specimens (obtained from 5 NB localized patients) were analyzed after mechanical disaggregation (Medi-machine; Becton-Dickinson Labware, San Jose, CA, USA).

During the same period, 10 BM obtained from 7 localized NB patients, 10 BM from localized Ewing sarcoma patients and 4 from non-Hodgkin's lymphoma (NHL) patients were used as a negative control. The negativity of these BM was established *via* routine morphological and immunocytochemical (ICC) analysis. In particular, no CD99, FLI1 or NB84 immunoreactivity was recorded on the 10 BM from the Ewing sarcoma patients, nor was NB84 immunoreactivity recorded on the localized NB BM. In addition, no neoplastic cells were recorded in 4 BM obtained from lymphoma patients. The parents' informed consent was obtained in all these cases.

Morphological evaluation. Each of the two aspirates in EDTA was smeared onto at least two slides and stained with May-Grunwald-Giemsa (MGG) for morphological evaluation. The BM biopsies were fixed, paraffin-embedded, and routinely stained with hematoxylin and eosin (H&E). The BM was scored as positive if at least 1 out of 4 sites revealed neoplastic cells.

FC evaluation. Mechanically-resuspended whole marrow aspirates (100 μl) were placed in a 12x75 mm round-bottomed polystyrene tube (Falcon 2054; Becton Dickinson Labware), fixed, permeabilized and stained using a Fix & Perm kit (Caltag Laboratories; Burlingame CA, USA), according to the manufacturer's instructions. Cells were incubated for 30 min at 4˚C with 10 μl of NB84FITC (clone NB84a/Ms; Caltag), CD56PE (clone MEM-188; Caltag) and CD45 Tri-color (R-PE Cy5 Tandem, clone HI30; Caltag). Finally, the samples were washed and resuspended in 500 μl PBS. Cells were analyzed on a FACS-Scan flow cytometer (Becton-Dickinson), considering at least 10^5 events. Isotype-matched controls optimized for intracellular staining (RCMG 106, RCGM 4993 and RCGM 4992, Caltag) were used to determine non-specific binding. The threshold was set on Forward Scatter (FSC) and the cell cytogram was verified on an FSC *vs.* Side Scatter (SSC) dot-plot. A capture area (R1) was drawn on a CD56PE *vs.* CD45TC dot-plot to include all CD56+/CD45– events. Events falling into R1 were gated and evaluated for FSC *vs.* SSC dot-plot, and boxed into R2 region. R2 events were then backgated and evaluated for NB84FITC expression on a monoparametric histogram plot. Only the cells falling in the R1 and R2 (logical gate operator AND) regions were considered as true NB cells and their number was used to calculate the final percentage of all BM white cells.

Spiking experiments. Normal EDTA whole blood with a known number of leukocytes was spiked with graded amounts of the NBT SK-N-BE cell line. Briefly, SK-N-BE cells were serially diluted into whole blood to represent abnormal cell frequencies ranging from 0.0001% to 10% of leukocytes. The staining and analysis procedures were the same as those described above; measured and predicted cell frequencies were compared to establish the assay's efficiency (Figure 2).

All the cell lines used for the present study, were kindly provided by Dott. M. Rodolfo, Department of Experimental Oncology, INT, Milan, Italy.

Statistical methods. In order to analyze the prognostic impact of BM infiltration (NB cells at diagnosis detected by FC), the patient's survival probability with or without BM involvement was evaluated using the Kaplan-Meier method (11) and the two curves were compared using the log-rank test.

Results

All the NB cell lines showed CD56 and NB84 co-expression (Figure 1, a1-a3). All 5 NB surgical specimens showed

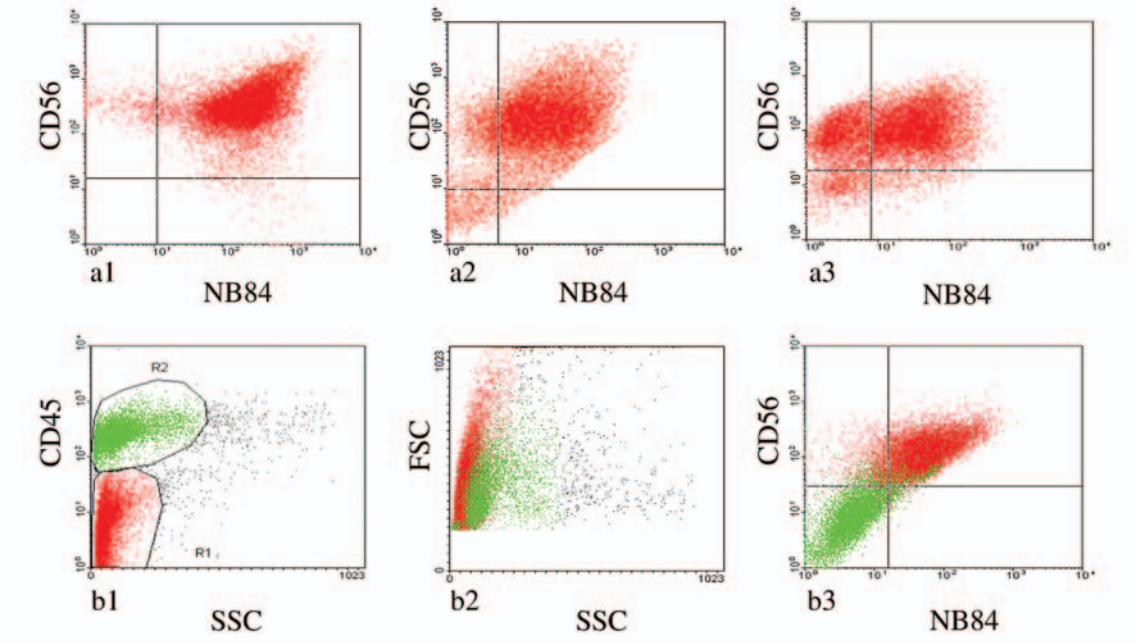

Figure 1. *The CD56+/NB84+ co-expression of neuroblastoma cell lines. a1: SK-N-BE (90%), a2: LAN 28 (80%) and a3: SK-N-5y (60%). The co-expression of CD56 and NB84 were also evaluated in IMR-32 (30%) and IMR-5 (10%), whereas the Ewing SK-N-MC cell line did not display NB84. The CD56+/NB84+/CD45– phenotypes of one surgical specimen: The FSC/SSC (b2) and CD56+/NB84+ (b3) properties of the CD45– cells (b1) are shown. The R2 region contains the CD45+ (hematological) component of the specimen.*

viable tumor after histological evaluation of frozen sections. A high degree (>90%) of CD56 and NB84 co-expression was observed in the CD45– cells obtained after completely disruption. The median fluorescence intensity was between 10^2 – 10^3 for both antibodies (Figure1, b1-b3).

The sensitivity threshold, obtained by serially diluting known amounts of SK-N-BE cells in peripheral blood, was established at 0.001% (10 SK-N-BE cells diluted in 10^6 peripheral blood cells, Figure 2).

Analyses conducted on 14 BM samples obtained from non-NB age-matched patients (10 with localized Ewing sarcoma and 4 with non-Hodgkin's lymphoma) occasionally revealed BM CD56+/NB84+/CD45– cells at the level of 0.001%; although the sensitivity of the assay was 0.001%, the specificity was 0.01%. Due to this fact, only the samples that displayed at least 0.01% CD56+/NB84+/CD45– events were considered as positives. The analyses conducted on 10 BM samples obtained from 7 localized NB patients (3 in stage 1 and 4 in stage 2) did not reveal CD56+/NB84+/ CD45–.

Dot-plots obtained from the BM drawn from one stage 4 and from one localized NB patient are shown in Figure 3. The CD56PE- and NB84FITC-fluorescence revealed by marrow CD45– cells was between the second and third decades on a four-decade scale of intensity. Microscopic analysis of MGG-stained smears confirmed the FSC/SSC physical features of the CD56+/NB84+/CD45– cells, which were small- to medium-sized mononuclear cells.

We evaluated the percentage of BM involvement at diagnosis in 27 NB stage 4 patients with a median age of 44 months (13-199, Table I). Briefly, 18 out of 27 had positive lymph nodes and 25 out of 27 had skeletal involvement. The two patients without skeletal involvement had metastatic lymph nodal disease (numbers 3 and 5, Table I). On morphological evaluation, BM aspirates were positive in 17 out of 27 (63%) cases (the BM was scored as positive if at least 1 of 4 sites revealed neoplastic cells); all these samples were also found to be positive using FC. Two further cases that were negative on morphological assessment were found to be positive using FC (numbers 9 and 10, Table I). Taken together, 19/27 (70%) BM samples were infiltrated by CD56+/NB84+/CD45– cells. The median percentage of BM NB cells was 2.5% (0.02% to 65%, Table I). The overall survival (OS) analysis showed that patients (n=8) without, or with <0.01% NB cells with FC had a significantly better outcome than patients (n=19) whose marrow revealed NB cells with FC ($p<0.05$, Figure 4).

Discussion

BM metastases are a gloomy prognostic sign in patients with NB stage 4 (1). CD56 and CD45 (in a triple-color FC assay) have been used with CD81 or CD9 in published reports for a more accurate detection of NB cells (8, 9). In the present study, NB84 was combined with CD56 and CD45 in a triple-

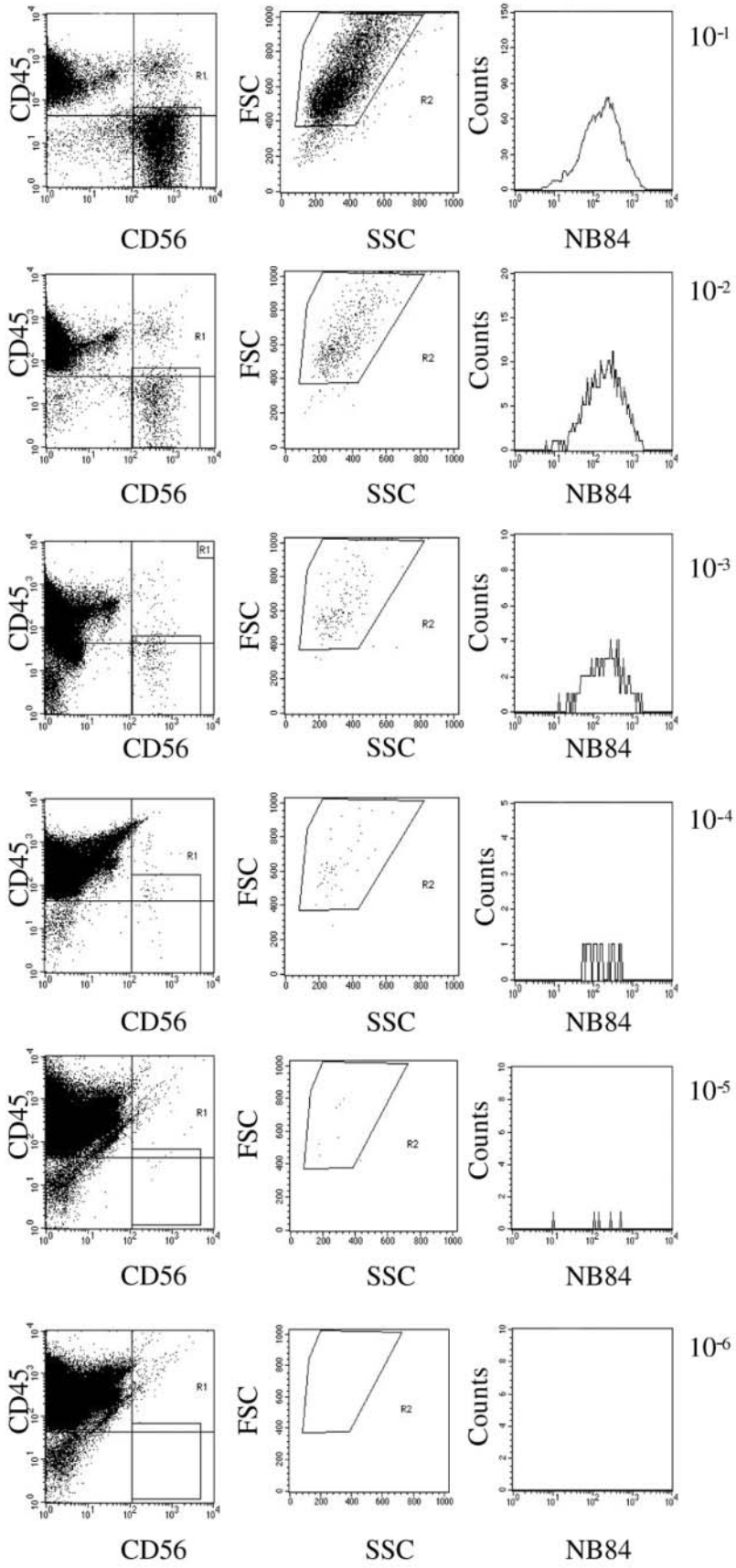

Figure 2. *Sensitivity of triple-color flow cytometric analysis. SK-N-BE cells were diluted stepwise from 1:10 to 1:10^{-6} with normal whole blood cells. The analysis was conducted as explained in the Methods. Regression analysis was performed: CD56+/NB84+/CD45-: log(y) = 0.05 + 1.09 log(x), R^2 0.99, standard error of the slope 0.02.*

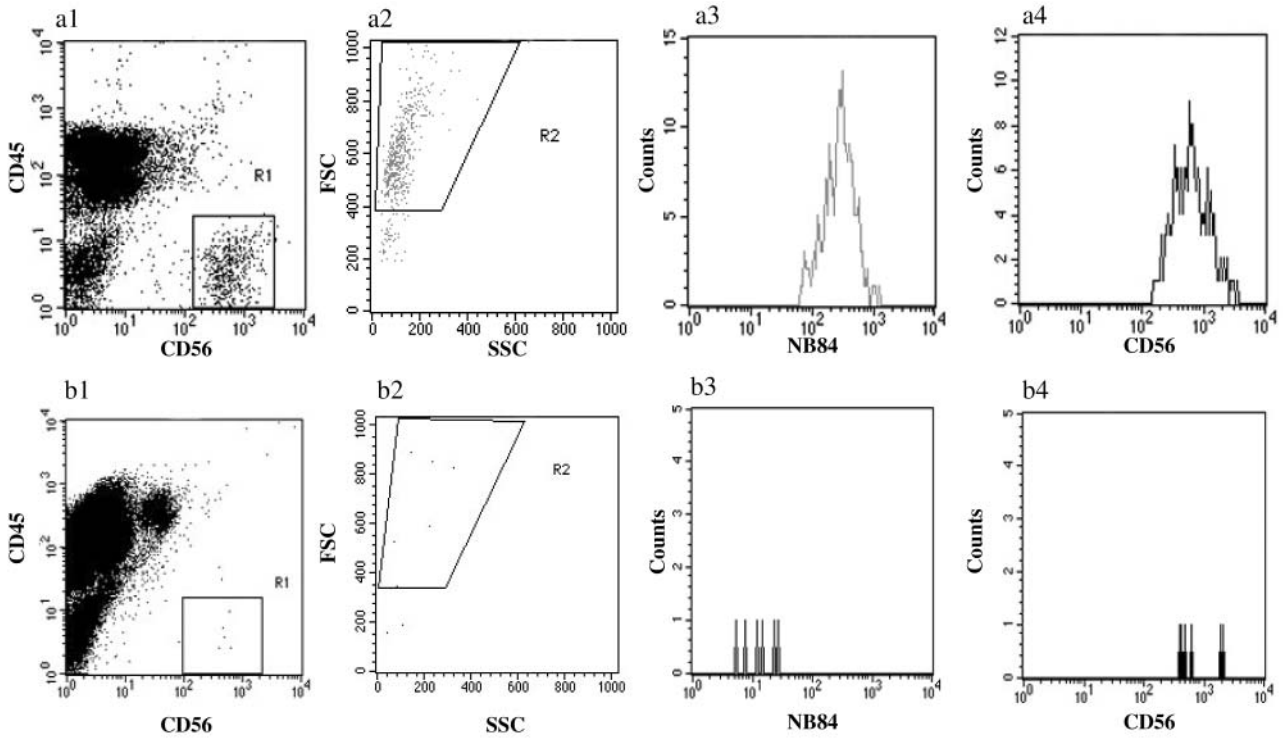

Figure 3. *FC analyses of BM samples of NB stage 4 (a1-a4, 2% of CD56+/NB84+/CD45– cells) and from one localized NB (b1-b4). The FSC/SSC properties and intensities of immunofluorescence were similar in the NB cells obtained from the surgical specimen (Figure 1, a4). Background staining (0,001%) was seen in the BM from the localized patient (stage 2).*

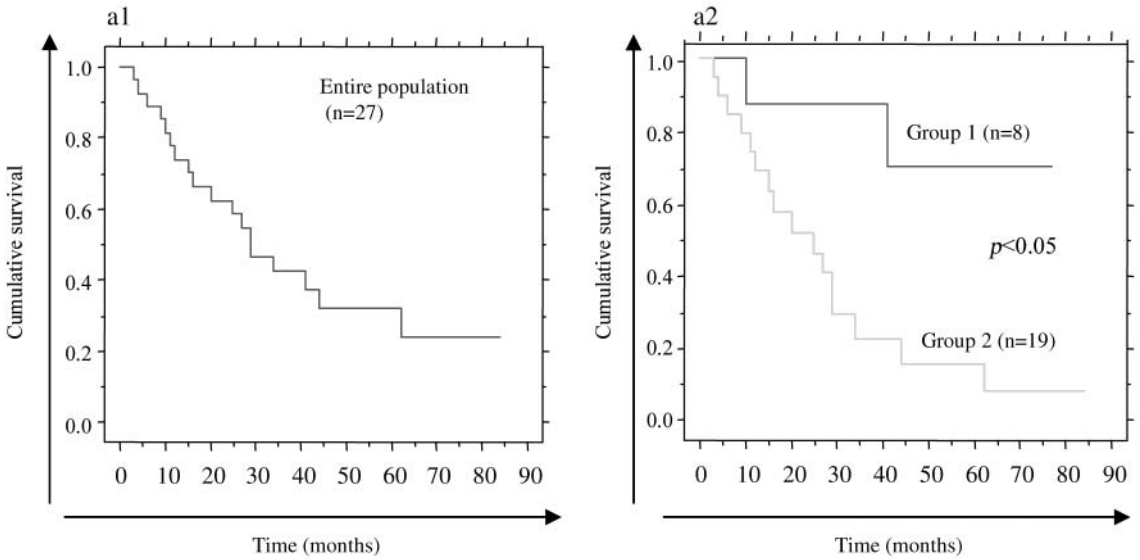

Figure 4. *Overall survival of the patient population. a1: series of patients as a whole (n=27). a2: according to NB cell detection in bone marrow using FC: group 1 (n=8) patients with negative bone marrow (blue line), and group 2 (n=19) patients with positive bone marrow (red line).*

color FC assay and the results were matched against conventional cytomorphology. First, the CD56+/NB84+/CD45– FC assay was tested on NB cells lines (Figure 1, a1-a3) and 5 NB specimens (Figure 1, b1-b3). In all cases, a clear-cut NB84 expression was apparent. This is consistent with the reported specificity (10) of NB84 (90%) and suggests that FITC-conjugated NB84 can be used for FC assays. Through spiking experiments, the sensitivity

threshold was established at 0.001% (Figure 2), but a cut-off at 0.01% was introduced after the FC evaluation of 14 BM samples obtained from non-NB age-matched patients. No CD56+/NB84+/CD45– cells were recorded in 10 BM samples obtained from 7 localized NB patients.

The BM obtained at diagnosis from 27 consecutive patients with stage 4 NB was analyzed using this method; 19/27 BM (70%) samples showed CD56+/NB84+/CD45– cells (Table I). In cases of massive BM infiltration (>1%), NB cells were detected in the BM using both FC and marrow cytology in all cases. With FC, 5 patients showed a percentage of NB cells between 0.01% and 0.5%; among these, the 2 with minimal marrow infiltration (0.02 and 0.09%) were not detected using standard morphological analysis. These data suggest that our method can identify some patients with even minimal BM involvement (<0.050%) who may escape notice on routine morphological analysis. Interestingly, each of these 5 BM patients (numbers 9 to 13, Table I), revealed NB84+ sparse cells by ICC, confirming the feasibility of the FC method. As reported in the literature for other FC methods (12), our FC assays did not improve the sensitivity of ICC, but NB cell quantification was more objective and rapid than using ICC. In addition, the FC immunolabeling of naïve NB84 antigen should reduce the loss of sensitivity reported between primary tumor specimens and trephine biopsy (13). It is worth noting that the BM involvement found in the present study (70%) is similar to the percentage reported by Seeger et al (80%), obtained at diagnosis in the largest reported series of patients with NB stage 4 (1), in which five different antibodies were used for ICC BM evaluation.

As for the sensitivity of FC in detecting bone marrow-infiltrating NB cells, one other study has reported positivity in 14 out of 14 (14) NB stage 4 patients. The percentage of positive BM detected at diagnosis by our method seems lower (19 out of 27; 70%), but this may be due to the use of the cut-off value. In addition, the larger series of patients evaluated using FC in the present study might also help to explain the difference in BM detection rate.

Having established our cut-off value at 0.01%, the BM at diagnosis of our 8 negative patients may either be truly negative (genuine disease-free BM) or a false negative (NB cells <0.01% and thus escaping our FC assay). Five of these 8 patients negative are long-term disease-free survivors (numbers 1, 2, 4, 6 and 7, Table I) and one (number, Table I) is alive with disease. This latter patient had a gross primary tumor in the retroperitoneum and metastatic lymph node disease. The patient remains alive and well 7 years after diagnosis, with gross inoperable disease at the primary tumor site. The remaining 2 patients without FC evidence of BM involvement died of disease. One (number, Table I), had a fatal progression of the primary tumor and the other (number, Table I) had a recurrence in the BM and skeleton (thus, prompting speculation as to FC possibly having underestimated any BM infiltration at diagnosis).

When survival probability was evaluated by dividing patients into two groups, with and without BM infiltration using FC analysis, the survival probability was significantly better for the group without BM infiltration (log-rank test, $p<0.05$, Figure 4). This result, again, is consistent with Seeger *et al.*'s findings that NB patients without or with minimal BM involvement at diagnosis (1-99 NB cells/10^5 BM nucleated cells) had a better event-free survival rate than those with overt BM infiltration. Although the limited number of cases makes it impossible to draw any final conclusions, we suggest that the 0.01% threshold detected using FC might be considered a conservative threshold for identifying NB stage 4 patients with "*very*" high-risk disease. In fact, despite the literature on micrometastatic disease in NB (detected using nested PCR), the relationship between micrometastases and recurrence is not yet fully understood (15). Although only using univariate analysis, to our knowledge, this is the first work in which the relationship between OS and the percentage of NB BM cells at diagnosis (detected using FC) was demonstrated in a homogenous and group of NB patients undergoing the same treatment. In conclusion, by combining CD45 and CD56 with the more specific NB84 antibody, we developed a rapid and reliable FC assay that can be associated with conventional cytomorphological BM evaluation to detect NB infiltration, especially in cases of dubious positivity. In addition, considering its clear cut expression, NB84 could be considered in a "specific" NB-panel with anti-GD2 (12, 16) for a more accurate assay for disease detection at diagnosis, during therapy and follow-up of NB stage 4 patients.

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