Combined Automatic Immunological and Molecular Cytogenetic Analysis Allows Exact Identification and Quantification of Tumor Cells in the Bone Marrow

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ABSTRACT

Purpose: **To improve the detection of disseminated tumor cells in bone marrow (BM) and peripheral blood samples of solid tumor patients, a novel computer-assisted scanning system for automatic search, image analysis, and repositioning of these cells was developed. This system allows precise identification and quantification of tumor cells by sequential immunological and molecular cytogenetic analysis. In this study, we attempt to demonstrate the practical use of this approach by analyzing BM samples from neuroblastoma patients.**

Experimental Design: **The disialo-ganglioside (GD2) molecule was used as the immunological target. The GD2 molecule was described as being specific for neuroblastoma cells, although false positive reactions had been suspected. To verify or disprove the neoplastic nature of the immunologically positive cells, sequential fluorescence *in situ* hybridization was performed on these cells to search for those genetic aberrations found in the corresponding primary tumors. A total of 115 samples from 40 newly diagnosed patients were evaluated for the presence of $GD2^+$ cells in the BM.**

Results: **GD2 positivity was detected in 95.2% of stage 4 patients, in 100% of stage 4s patients, and in 38.5% of patients with localized/regional disease. In stage 4 and 4s BM samples, the $GD2^+$ cells were unequivocally identified as tumor cells based on the molecular cytogenetic aberrations found by fluorescence *in situ* hybridization. However, in BM samples from patients with localized/regional disease, all $GD2^+$ cells were concluded to represent false positivity due to the absence of genetic aberrations.**

Conclusions: **Automatic search and sequential molecular cytogenetic analysis of the immunologically positive cells provide precise information on both the number and cytogenetic profile of disseminated tumor cells.**

INTRODUCTION

NB,[2] the most frequent solid neoplasm in young children, originates from the sympathetic nervous system. In localized/regional (stages 1–3) and stage 4s disease, predominantly numerical chromosomal aberrations have been described. Advanced disease (stage 4) is, in addition, frequently associated with characteristic structural cytogenetic aberrations (1, 2). Deletion at the short arm of chromosome 1 [del(1)(p36)], amplification of the *MYCN* oncogene, and/or gain of the long arm of chromosome 17q (3–6) are genetic changes that are supposed to be signs of aggressive tumor behavior.

Reliable detection of tumor cells in BM samples is fundamental in the initial staging and for the monitoring of therapy response (minimal residual disease) and is presumably also of great prognostic significance. Furthermore, the lack of detectable disseminated tumor cells in stem cell products significantly contributes to the success of autologous BM transplantation in advanced NB (7, 8). Therefore, highly specific, sensitive, and quantitative methods are increasingly required for the effective analysis of disseminated tumor cells in hematopoietic samples.

Today, disseminated NB cells are detected by classical cytomorphological examination in clinical practice (9). Unfortunately, the lack of high sensitivity, difficulties in quantification, and interobserver differences clearly reduce the power of this method of detection. The identification of NB-related gene transcripts, such as tyrosine hydroxylase (10, 11) or GAGE (12), has been successfully applied using reverse transcription-PCR. However, the relative quantification of cell type-associated RNA transcripts in diagnostic materials has proven to be complicated and very often impossible.

The immunological detection of antigens expressed by NB cells has been successfully applied for the sensitive demonstration of isolated tumor cells in the BM (13–16). Unfortunately, the evaluation of microscopical preparations is time-consuming and complicated by numerous methodological issues (17). Enzymatic detection systems, for instance, may lead to unspecific reactions mimicking positivity due to endogenous enzyme activity (18). Because immunological analysis can result in dubious positive findings, unambiguous features disclosing the true nature of the immunologically positive cells should be considered.

We will present an automatic approach to detect and quan-

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[2] The abbreviations used are: NB, neuroblastoma; GD2, disialo-ganglioside; FISH, fluorescence *in situ* hybridization; MNC, mononuclear cell; DAPI, 4′,6-diamidino-2-phenylindole; BM, bone marrow.

tify NB cells in the BM, using immunofluorescence labeling by a monoclonal antibody specific for ganglioside GD2 (19). The fluorescence microscope-based automatic image analysis system allows a consecutive analysis of cytogenetic aberrations by FISH of the selected cells (20). Previous spiking experiments clearly revealed that not all immunologically positive cells displayed the "tumor typical" cytogenetic aberrations. These cells did not show any genetic aberrations by FISH and therefore had to be considered as falsely positive cells (21). We analyzed routine BM samples from newly diagnosed NB patients to demonstrate the power and the practical use of the sequential immunological and FISH approach in a clinical setting.

MATERIALS AND METHODS

Cell Preparation. The MNC fractions of heparinized BM aspirates from 1–4 punction sites of 40 newly diagnosed NB patients were isolated by Lymphoprep (Nycomed, Oslo, Norway) gradient centrifugation and washed two times in RPMI 1640 containing 10% FCS. In addition, BM specimens from 10 individuals without NB were analyzed as control samples in the same way. Large cytocentrifuge preparations (240 mm^2), optimally containing 0.5 to 10^6 MNCs/slide in a volume of 500 μl were prepared on silanized glass slides (3000 rpm, 6 min) with a Hettich cytocentrifuge (Hettich, Tuttlingen, Germany) and air dried.

Immunofluorescence Analyses. The cells were fixed in 4% formaldehyde/PBS for at least 1 h at 4°C. Before the immunofluorescence staining, slides were incubated with 4% BSA to block unspecific antibody binding for 20 min at 37°C. Slides were incubated for 30 min with an unconjugated mouse monoclonal antibody against the GD2 antigen (monoclonal antibody 14.18; 1:500 dilution; kindly provided by R. A. Reisfeld; The Scripps Research Institute, La Jolla, CA). Detection of the antibody was done using a FITC-labeled goat antimouse antibody (30 min; 1:60 dilution; DAKO, Glostrup, Denmark). Slides were covered with the antifade medium Vectashield (Vector Laboratories, Burlingame, CA) containing DAPI (1 μg/ml).

FISH. Coverglasses were carefully detached, rinsing the slides in PBS. Residual immunofluorescence was then eliminated by proteinase digestion [50 μg/ml pepsin (pH 1.5)] for 1–10 min. *In situ* hybridization was performed with either of the pairs of DNA probes to determine the integrity of 1p36.3 (D1Z1 and D1Z2), *MYCN* status (N-myc and D2Z), or gain of 17q material [D17Z1 and D17S881-2; obtained from American Type Culture Collection and Oncor Inc. (Gaithersburg, MD)]. Cells and probes were denatured on a heating plate together at 80°C for 10 min. Overnight hybridization at 37°C was followed by two washes in 2× SSC containing 50% formamide for 7 min at 42°C and two washes in 2× SSC. The probes were detected with mouse antibiotin (DAKO) and FITC-conjugated sheep antidigoxigenin antibodies (Roche, Vienna, Austria), followed by a second incubation with tetramethylrhodamine isothiocyanate-conjugated antimouse and FITC-conjugated antisheep antibodies (DAKO). The slides were covered with Vectashield-DAPI. Sequential FISH analyses were performed in up to three different hybridization reactions with each of the above-listed pairs of DNA probes to prove the presence of multiple genetic aberrations within the same tumor cell nuclei. These additional tests were performed to determine whether the immunologically positive cells featured the same cytogenetic markers found in the primary tumor.

Scanning Procedure and Cell Identification. Slide scanning, cell identification, storage of the exact coordinates on the slide, and the image as well as quantification of cells and repositioning were done with the help of the Metafer 3.0 automatic fluorescence image analysis system equipped with RCDetect software (MetaSystems GmbH, Altlussheim, Germany). This fluorescence object-finding and relocation system is based on a motorized Axioplan2 fluorescence microscope (Zeiss, Göttingen, Germany), a motorized eight-slide scanning stage (Märzhäuser, Germany), a high-resolution charge-coupled device camera, and a personal computer equipped with appropriate cards for accurate stage movement and fast image analysis.

The automatic scanning procedure was performed as follows: DAPI-counterstained nucleated cells were automatically focused, and the number of the nucleated cells was determined (19). Then, the whole slide was scanned for specific immunofluorescence related to GD2 (FITC). The exact position on the slide (*X* and *Y* coordinates) and the color image (DAPI/FITC) of positive cells were stored. Both the number of positive cells and the total number of analyzed cells were given in absolute figures.

Target Cell Repositioning and FISH Evaluation. The sequential evaluation of the FISH results was done after replacing the slide in the stage and repositioning the cells selected on the basis of their positive immunofluorescence. After capturing the FISH image with the integrated ISIS software, a gallery of images with different immunological and corresponding FISH pictures was created.

Evaluation of Results. Samples containing at least 1000 strong $GD2^+$ cells/10^6 MNCs (0.1%), which also fitted the morphological criteria of neoplastic cells, were accepted as being positive without FISH verification. Slides with less than 0.1% $GD2^+$ cells were sequentially analyzed for at least one cytogenetic aberration found in the primary tumor. The neoplastic nature of a given cell could be determined when both the GD2 and the FISH positivity were demonstrated. $GD2^+$ cells without the typical cytogenetic aberration of the primary tumor were declared to be falsely positive and excluded from the evaluation.

RESULTS

BM aspirates from 40 NB patients at initial diagnosis and from 10 control patients were evaluated by an automated search for $GD2^+$ cells. The aspirates from 1–4 different punction sites from each individual patient gave a total number of 115 BM samples. The mean number of total BM MNCs analyzed per puncture site was $0.94 \times 10^6 \pm 0.2 \times 10^6$ SD (range between 0.18×10^6 and 3.2×10^6 MNCs). None of the slides from the control patients showed GD2-specific immunofluorescence. However, 31 of 40 patients (*i.e.,* 77 samples) were found to contain GD2-immunolabeled cells throughout all disease stages. Samples from 5 of the 13 patients with localized/regional NB

Table 1 BM findings according to the age, disease stage, and cytogenetic aberrations of the primary tumor of 41 NB patients identified by the automatic microscopic procedure

Tumor cell positivity was stated when the tumor typical cytogenetic aberrations were found in at least two GD2 immunolabeled cells by a targeted sequential FISH analysis after automatic repositioning ($GD2^+/FISH^+$).

Patient	Age[a]	Disease stage	Genetic aberration in the primary tumor	No. of punction	$GD2^+$ samples	$GD2^+/FISH^+$ samples
1	9 m	1	del1p	4	0	0
2	4 y	1	Trisomy 1 and 2	2	0	0
3	13 m	1	Pentasomy 1	4	2	0
4	3 m	2B	Trisomy 1 and 2	4	0	0
5	11 m	2B	del1p	2	0	0
6	11 m	2B	imb1p, 17q gain	4	3	0
7	4 y	3	17q gain	1	0	0
8	10 m	3	Trisomy 1 and 2	4	0	0
9	4 y	3	n.t.[b]	4	0	0
10	11 m	3	Tri- and hexasomy 1 and 2	2	0	0
11	3 y	3	*MYCN* ampl	4	3	0
12	12 m	3	17q gain	4	3	0
13	3 y	3	del1p, *MYCN* ampl	4	4	0
14	2 y	4	Tri- and tetrasomy 1 and 2	4	4	4
15	3 y	4	17q gain	2	2	2
16	12 y	4	17q gain	1	1	1
17	2 y	4	17q gain	1	1	1
18	2 y	4	imb1p	4	4	4
19	6 y	4	del1p	4	4	4
20	12 y	4	del1p	4	4	4
21	8 m	4	del1p	2	2	2
22	4 y	4	imb1p	1	1	1
23	15 m	4	del1p, *MYCN* ampl	3	3	3
24	14 m	4	del1p, *MYCN* ampl	4	0	0
25	3 y	4	del1p, gain *MYCN*	2	2	2
26	2 y	4	del1p, *MYCN* ampl	1	1	1
27	3 y	4	del1p, gain *MYCN*	1	1	1
28	4 y	4	del1p, *MYCN* ampl, 17q gain	2	2	2
29	2 y	4	del1p, *MYCN* ampl, 17q gain	1	1	1
30	4 y	4	imb1p, *MYCN* ampl, 17q gain	4	3	3
31	5 y	4	del1p, *MYCN* ampl, 17q gain	4	4	4
32	18 m	4	imb1p, *MYCN* ampl, 17q gain	4	3	3
33	5 y	4	del1p, *MYCN* ampl, 17q gain	1	1	1
34	2 y	4	del1p, *MYCN* ampl, 17q gain	1	1	1
35	2 m	4s	Trisomy 1	4	3	3
36	1 m	4s	Trisomy 1	4	3	3
37	10 m	4s	Trisomy 1 and 2	4	4	4
38	4 m	4s	Trisomy 1 and 2	2	2	2
39	5 m	4s	Tetrasomy 1	3	1	1
40	13 m	4s	Tri- and tetrasomy 17	4	4	4

[a] m, month; y, year.
[b] n.t., not tested.

(stages 1, 2B, and 3; 38.5%), 20 of 21 stage 4 patients (95.2%), and all 6 stage 4s patients (100%) patients displayed $GD2^+$ cells. The GD2 findings in relation to the stage of the disease are presented in Table 1.

In 28 BM samples, the GD2 positivity reached 0.1% or an even higher percentage. The specificity of the immunolabeling was controlled in the remaining 49 positive samples by sequential FISH reactions after repositioning the $GD2^+$ cells. Interestingly, the FISH analyses directed at the tumor typical cytogenetic aberrations did not support the neoplastic nature of the $GD2^+$ cells in any of the samples from patients with localized/regional NBs (Table 1). Some of these immunopositive cells cytomorphologically resembled macrophages or storing cells (Fig. 1*A*). However, they frequently appeared as small, round cells indistinguishable from tumor cells (Fig. 1*C*). After sequential FISH, all of these cells showed two normal hybridization signals with either of the DNA probes (Fig. 1, *B* and *D*). Because numerical chromosomal aberrations were homogeneously found in the primary tumor samples from the same patients by applying the same DNA probes, these $GD2^+$ cells were interpreted to be nonneoplastic and excluded from the evaluation. In summary, all BM samples containing isolated $GD2^+$ cells from stage 1, 2B, and 3 NB patients proved negative for tumor cells due to of the absence of tumor typical genetic aberrations.

On the other hand, most $GD2^+$ cells from stage 4 and stage 4s NB patients that we analyzed sequentially by FISH displayed the expected numerical or structural cytogenetic aberrations. In 19 of 20 BMs from stage 4 patients with $GD2^+$ cells, del(1)(p36)/imb(1)(p36), *MYCN* amplification, and/or gain of 17q could be proven. Moreover, in seven of these patients (patients 28–34, Table 1), sequential FISH detected all three

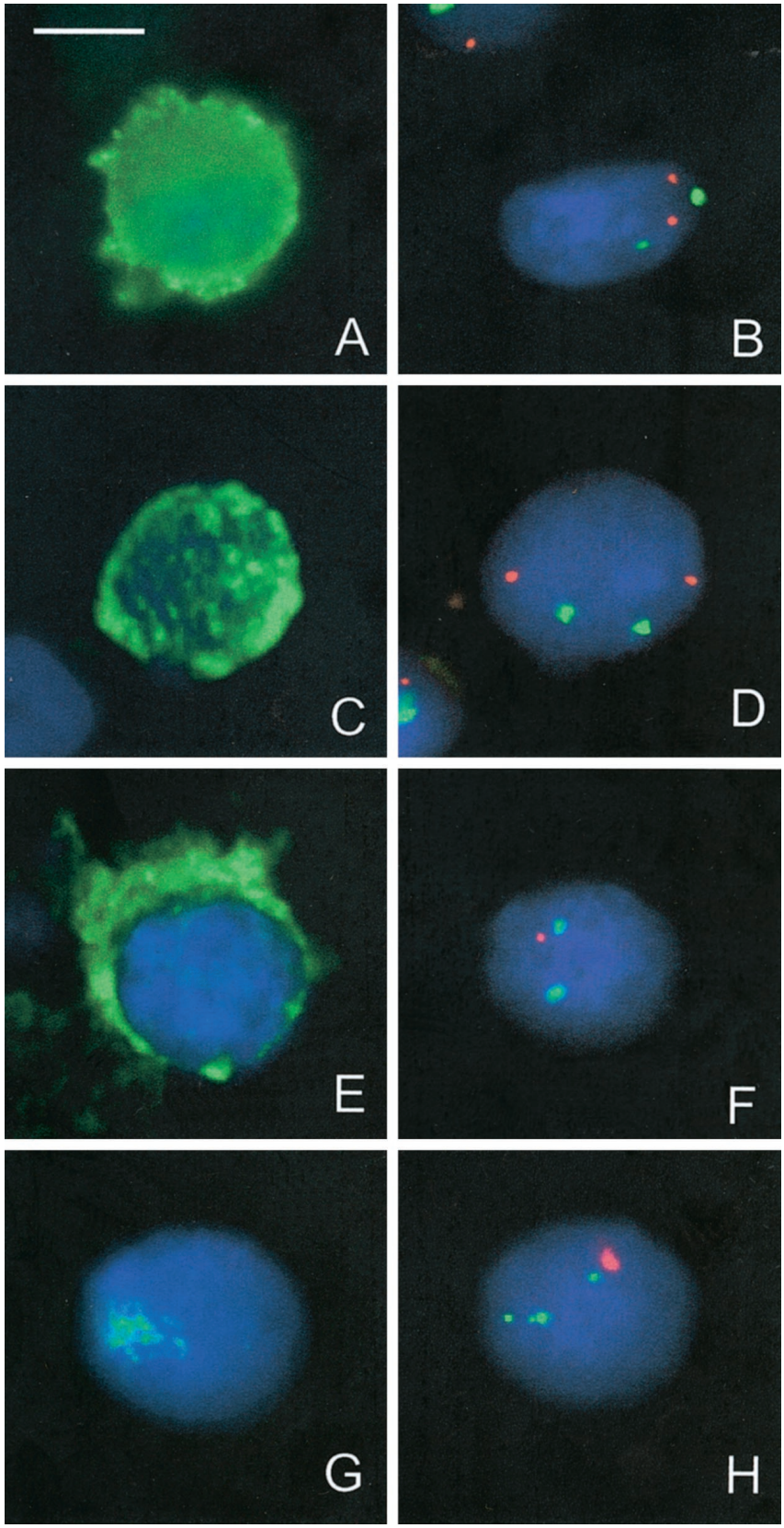

Fig. 1 False GD2 immunofluorescence labeling in a macrophage-like cell (*A*) and in a small round cell resembling a tumor cell (*C*) in the BM of a stage 3 NB patient. Two *red* (D1Z2) and two *green* (D1Z1) fluorescence signals (*B* and *D*) representing a disomy of chromosome 1 in these cells could be detected by sequential FISH analysis after automatic repositioning, whereas a clear trisomy 1 was demonstrated in the primary tumor. Genetic profile of a GD2 immunolabeled (FITC) disseminated NB cell (*E*) by sequential FISH analysis: one *red* (D1Z2) and two *green* (D1Z1) fluorescence signals referring to a del(1)(p36) are seen (*F*); numerous *green MYCN* signals due to a ~20-fold amplification of the *MYCN* gene are seen (*G*); and four *green* signals specific to 17q (D17Z881-2) and one *red* signal of the centromeric region of chromosome 17 (D17Z1) demonstrating a gain of 17q (*H*) are seen.

Table 2 Number of patients displaying the given amounts of $GD2^+$ and tumor cells in the analyzed 10^6 MNCs

The frequency of $GD2^+$ and $GD2^+/FISH^+$ tumor cells in BM samples of 40 NB patients according to the disease stage. The number of samples displaying the respective number of tumor cells/10^6 MNCs is given.

	No. of patients with 0 $GD2^+$ cells[a]	No. of patients with 1–9		No. of patients with 10–99		No. of patients with 100–999		No. of patients with 1000	
		$GD2^+$ cells[a]	Tumor cells[b]	$GD2^+$ cells[a]	Tumor cells[b]	$GD2^+$ cells[a]	Tumor cells[b]	$GD2^+$ cells[a]	Tumor cells[b]
Stage 1	2	1	0	0	0	0	0	0	0
Stage 2A, B	2	1	0	0	0	0	0	0	0
Stage 3	4	3	0	0	0	0	0	0	0
Stage 4	1	4	4	2	2	0	0	14	14
Stage 4s	0	3	3	2	2	0	0	1	1
No. of patients/total	9/40	12/40	7/40	4/40	4/40	0/40	0/40	15/40	15/40

[a] Cells with GD2-specific surface immunofluorescence positivity.
[b] Cells positive for both GD2 and tumor typical cytogenetic aberration (FISH).

structural chromosomal aberrations that were also demonstrated in the primary tumors using the same DNA probes (Fig. 1, *E–H*). In the remaining positive stage 4 patient and in all stage 4s patients, tri-, tetra- or pentasomies of chromosomes 1, 2, or 17 were demonstrated in the primary tumors and served as markers to verify disseminated tumor cells in the BM using the adequate DNA probes.

In 1 of the 21 stage 4 patients (case 24, Table 1), a negative initial BM finding was obtained. In the slides from these samples, however, only a mean number of 0.4×10^6 MNCs ($\pm 0.1 \times 10^6$, SD) were available for analysis. Thus, less than half of the total number of cells was present as compared with the mean number of analyzed cells in the other cases.

Table 2 demonstrates the quantitative evaluation of patient positivity with respect to clinical stage. In localized/regional disease (stages 1–3), sequential FISH analysis of $GD2^+$ BM cells had a major influence on BM positivity but was found to have no effect on stage 4 and 4s BM findings. After the elimination of false GD2 positivity by FISH occurring in early-stage disease (stages 1–3), the rate of tumor cell-positive patients was shown to decrease by 19%, and the rate of positive samples analyzed in this study was shown to decrease by 21%.

The median values of tumor cells present in the BM of stage 4 and stage 4s patients were 550 and 3 per 10^6 MNCs, respectively. This difference in the tumor cell concentration between the two stages statistically proved to be highly significant ($P < 0.001$). A tumor cell load of 0.1% or higher was demonstrated in 57.7% of the stage 4 patients but in only 5.9% (one patient) of the stage 4s patients. On the contrary, 33.3% and 94.1% of the samples showed low tumor cell contamination (<100 tumor cells/sample) from stage 4 and stage 4s patients, respectively (Table 2).

DISCUSSION

In recent years, several immunological and molecular approaches to demonstrate disseminated tumor cells in hematological samples from solid tumor patients have been published. The immunological detection of tissue-specific antigens by enzymatic procedures, *e.g.*, myeloperoxidase-conjugated or alkaline phosphatase-conjugated secondary antibodies, has been frequently applied. Using these methods, isolated tumor cells were described in the BM of patients with localized/regional disease stages (stages 1–3). In addition, a prognostic impact of disseminated tumor cells was proposed in patients with localized epithelial cancers and NB (15, 22–24). However, recent observations underline the limitations of the enzymocytochemical detection systems because they may result in false positive staining due to endogenous enzyme activity in hematopoietic cells (17, 18).

In this study, a quantitative method for the demonstration of NB cells is introduced using an immunofluorescence-based automatic microscopic scanning approach with consecutive verification of the neoplastic nature of the respective cells. The cell surface ganglioside GD2 is one of the most reliable immunological markers for NB (25, 26), and it serves as an ideal target for automated image analysis. The search method used in our study is based on characteristic fluorescence parameters of the immunolabeled cells. It is performed field by field on the slide, which is followed by storage of the position and the digital image of all positive cells. If necessary, each individual immunopositive cell can subsequently be analyzed morphologically and/or by sequential FISH (20, 21). Not only does fluorescence microscopy represent a simple way to determine isolated tumor cells, but it also makes it easier to count the nucleated cells in the sample, which results in a highly accurate tumor cell quantification (19).

With spiking experiments, we could demonstrate that even 1 cytogenetically proven NB cell in 10^6 MNCs can be recovered unequivocally (21). In these spiking experiments, however, the number of $GD2^+$ cells constantly exceeded the number of the spiked tumor cells. FISH to display tumor typical genetic aberrations clearly demonstrated that these cells were of a nontumorous nature. Therefore, one of the most likely explanation of false positive reactions could be a transloading of the tumor cell-derived ganglioside to hematopoietic cells after physical contact with $GD2^+$ tumor cells. Furthermore, the presence of apoptotic cells, which were shown to be positive for tumor-specific markers (27), has also been implied. These are expected, in both an isolated and an internalized form, to result in sample positivity with an unclear impact on the rate of viable disseminated tumor cells.

In the present study, the vast majority of the BM samples

from stage 4 and stage 4s patients and more than one-third of the BM samples from patients with stage 1, 2B, and 3 NB displayed $GD2^+$ cells. The morphological distinction between specific and unspecific GD2 immunolabeling was found to be especially difficult or even impossible when only a few positive cells were found in the BM sample. Even careful morphological analysis after automatic relocation could not support or exclude the true nature of individual $GD2^+$ cells. In all unclear cases, however, at least one tumor typical cytogenetic aberration was demonstrated in the primary tumors that could be specifically applied to clarify the nature of the $GD2^+$ cells in the BM. Genetic differences between primary tumor and disseminated tumor cells could also be addressed applying automated relocation. In samples from stage 4 and stage 4s patients, sequential molecular cytogenetic analysis of *MYCN*, 1p, and 17q in the same disseminated tumor cells always presented the same aberrations as found in the primary tumor. This finding strongly supported the idea that a targeted analysis of cytogenetic aberrations can be efficiently used to detect rare tumor cells.

Interestingly, none of the GD2 immunofluorescence-positive cells in the BM samples of patients with localized/regional disease displayed numerical chromosomal aberrations characteristic of the individual primary tumors. Therefore, the neoplastic nature of these immunofluorescence-positive cells could be excluded. The false positive rate concerned 38.4% of the patients in this disease group. It is noticeable that previously published results demonstrated GD2 positivity in the BM in a similar number (*i.e.*, in 34%) of patients with localized/regional NBs by immunocytochemistry using the myeloperoxidase detection method (15). The positive cells in the study of Moss *et al.* (15) were interpreted as tumor cells without further clarification of their biology. However, endogenous enzymatic activity, active uptake of GD2 by macrophages, and the peculiar transloading capacity of the GD2 molecule described here may all result in false positive findings. The use of immunofluorescence for the demonstration of disseminated tumor cells, on the contrary, offers the following advantages: the likelihood of endogenous enzymocytochemical reactions is eliminated; and immunologically selected doubtful cells can be scrutinized.

Another remarkable finding in this study was the difference in the quantity of tumor cells between stage 4 and stage 4s BM samples. Stage 4s NB, despite the highly proliferative initial phase and fast spreading, generally has a favorable outcome. Despite the different clinical behavior, the tumor cell positivity rate was found to be similar to that in stage 4 patients. Significantly lower numbers of tumor cells in the BM of patients with stage 4s disease can possibly be used to demonstrate a less aggressive disease behavior, although we have used only a small patient collective.

In 18.2% of all analyzed samples, which represent 33.8% of all positive samples, less than 10 tumor cells/10^6 MNCs were found, indicating that automatic fluorescence microscopy is a powerful approach for the detection of rare tumor cells. Major critical issues of the rare cell detection, *i.e.*, the exact quantification of the tumor cell infiltrate and the presence of false positivity, can also be overcome by this application. In addition to the verification of their neoplastic nature, molecular cytogenetic analysis allows further insights into the biology of disseminated tumor cells.

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