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Immunological Detection and Definition of Minimal Residual Neuroblastoma Disease in Bone Marrow Samples Obtained During or After Therapy

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Abstract—*Immunological staining by the alkaline phosphatase/anti-alkaline phosphatase (APAAP) technique has been used to recognize low levels of neuroblastoma cells in bone marrow mononuclear cells. Immunological phenotyping with 11 well characterized monoclonal antibodies was performed on 16 children with neuroblastoma and BM involvement during or after therapy. Neuroblasts were detected in 11 of 16 patients (0.1–5%), whereas BM biopsies on six of these patients were classified as normal. Aspirates, stained conventionally, were positive for pathological cells in three patients only.*

The comparison of the phenotype of the neuroblastoma cells at the time of diagnosis to the phenotype of the residual cells within one patient revealed differences. The phenotype of residual disease in different patients on the other hand showed a unique pattern. The above mentioned results lead to the conclusion that the immunological procedure is particularly suitable for the analysis of minimal residual neuroblastoma since the technique allows very minor cell populations to be identified in BM samples.

INTRODUCTION

THE ACCURATE ASSESSMENT of tumour infiltration in bone marrow from patients with neuroblastoma yields important clues for their optimal management. Although cell surface marker expression, cytogenetic analysis and more recently the study of N-myc oncogene amplification have been used to characterize neuroblastoma [1–3], the standard method for determining the presence of tumour cells in bone marrow still remains the examination of bone marrow aspirate and biopsy specimens by light microscopy. All of these techniques have variable sensitivity for the detection of small numbers of neuroblastoma cells and the presence of clonal evolution within a malignant population. Since autologous bone marrow transplantation is now the treatment of choice for advanced neuroblastoma [4, 5], the detection of occult tumour cells in the bone marrow may have great significance.

The fact that the study of N-myc oncogene amplification and cytogenetic studies does not permit a single cell analysis makes immunological detection of neuroblastoma cells the method of choice in the study of neuroblastoma minimal residual disease. Two recent papers describe a combination of conventional cytological methods with immunological detection of the remaining cells using a rather restricted panel of monoclonal antibodies (HSAN-1, UJ 13A and UJ13A, 5A7), which do not allow detection of neuroblasts below 5% [6, 7].

However, no study has attempted to identify and define these minor cell populations with a technique that will maximize tumour cell detection without overloading the laboratory. We chose the APAAP technique, since its simplicity and the clarity with which it identifies different cell populations suggest that it could be an ideal means of studying neuroblastoma minimal residual disease [8].

Using a recently described panel of monoclonal antibodies which distinguish neuroblasts from haemopoietic cells [9], we were able to identify neuroblastoma cells below 1%.

PATIENTS AND METHODS

Fourteen patients with neuroblastoma stage IV and two patients with stage IV-S (diagnosed according to classical criteria previously reported [10]) were included in this study. Investigations were performed during or after therapy (Table 3 shows the treatment and the clinical response at the time of immunological analysis as well as the clinical

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outcome studied in a median follow-up time of 1.5 years).

All patients had an initial BM involvement. In seven patients an immunological analysis was done at time of diagnosis and the phenotype of initial NB disease in BM was compared with the phenotype of NB minimal residual disease (see Table 6). In order to define the specificity of the antibodies on bone marrow cells immunological analysis was performed on four patients without bone marrow disease and on eight patients with acute leukaemia in remission. The results are shown in Table 2.

BM samples and immunological technique

Four biopsies and four aspirates were taken anteriorly and posteriorly from both iliac crest in all patients. BM aspirates from each side were pooled into heparinized bottles, a mononuclear cell fraction was prepared by centrifugation on Ficoll and cells adjusted to 2×10^6/ml in PBS. Cells were transferred onto poly-L-lysine (Sigma, Munich, F.R.G.) coated spots on glass slides. Using a high molecular weight polymer of L-lysine as a slide coating, cell adhesion was improved and preparation of cytospins could be avoided. Each glass slide was divided into 10 separate fields of reaction. In each field of reaction approximately 10,000 cells are found. From each aspirate we examined two slides. From each field of reaction on the slide at least 500 cells were counted in order to estimate the positively reacting cells. After 30 min the attached cells were fixed for 90 s in buffered formal acetone and labelled with the APAAP technique as described previously [11]. Trephine biopsies obtained with a Jamshidi needle were fixed on formalin and afterwards stained with standard methods.

Monoclonal antibodies

The MAbs employed in this study are detailed in Table 1. The MAbs UJ 13A, UJ 127.11, UJ 181.4, UJ 167.11, M1N1, A2B5, PI 153/3 and anti-Thy-1 were kindly provided by Dr J.T. Kemshead (Imperial Cancer Research Fund Laboratories, Institute of Child Health, London, U.K.). Sheep anti-mouse immunoglobulins and monoclonal APAAP complexes are available from Dakopatts, Denmark as well as the Mab DAKO-NF (clone NR4), which reacts with the 68 kD component of the three major polypeptide subunits (68, 160 and 200 kD) generally present in neurofilaments. The MAbs BA1 and BA2 were purchased from Hybridect, San-Diego, CA, U.S.A.

RESULTS

Bone marrow mononuclear cell smears were stained by the APAAP procedure with 11 monoclonal antibodies against the neuroblastoma cell antigens listed in Table 1. The results are summarized in Table 3 and illustrated in Figs. 1–4. Table 2 shows the reaction of MAbs to normal BM cells and BM cells obtained from patients with haematological malignancies in remission.

All cases showed a clear distinction between positive and negative cells and there was no background labelling. Neuroblastoma cells stained in 11 patients both as clumps and single cells with an intense red colour (see figures). In BM taken from children without bone marrow disease and with other haematological diseases (in remission) some MAbs stain up to 1% (UJ 127.11, UJ13A, UJ181.4) and others stain more cells (M1N1, BA1, PI 153/3). In such cases, cells appear single and lymphoid-like whereas neuroblastoma cells are usually gathered in clumps. Samples were then classified as normal when fully negative, pathological when more than 2% of single cells were positive with at least two MAbs from which normal BM stain up to 1% (UJ 13A, UJ 127.11. UJ 181.4). Any stained clump of cells was considered to contain neuroblastoma cells.

In eight cases, cells as both clumps and single cells were detected. In three cases single cells only were detected.

A comparison of APAAP staining with conventional techniques (histology and cytology) led us to conclude that immunological staining of marrow with MAbs enables tumour detection with a specificity and sensitivity exceeding that of standard cytological and histological methods, since biopsies were positive in six cases while conventionally stained aspirates were positive in only three cases (see Table 4). These data are consistent with the findings of Bjork *et al.*, who could detect neuroblastoma cells in 30 of 37 marrows studied, whereas histology revealed tumour cells in only 18 cases [12].

In seven patients, a comparison between the initial NB phenotype and the phenotype of the residual disease was possible. It seems that the residual cells consist of a malignant clone with an unique phenotype. These cells reacted strongly with three MAbs (UJ 167.11, A2B5, UJ 13A) but showed no reaction with other MAbs which reacted with the initial NB cells (anti-Thy-1, BA2, BA1, UJ 181.4, UJ 127.11, anti-neurofilaments). We cannot, however, rule out antigens being lost during therapy, although this chemotherapy-induced antigen loss has never been observed.

No minimal residual disease could be detected in two cases of patients with stage IV-S disease.

DISCUSSION

One of the major problems associated with treatment and prognosis in neuroblastoma is to try to ensure that the marrow is free from tumour cells. As neuroblastoma cells appear, by conventional

Table 1. Details of monoclonal antibodies used

Antibody	Reactivity pattern	Source or reference
UJ13A	Neuroectodermal tissue and tumours	JT Kemshead
UJ127.11	Normal brain, neurones, neuroblastoma, schwannoma	JT Kemshead
UJ181.4	Foetal brain, medulloblastoma, neuroblastoma	JT Kemshead
UJ167.11	Cells of neural crest origin	JT Kemshead
M1N1	Neuroblastoma cells, granulocytic cells, some T-lymphoblasts, foetal and adult brain	Kemshead *et al.* [19]
A2B5	Normal brain and neuroblastoma cells	Kemshead *et al.* [20]
PI153/3	Neuroblastoma, c-ALL cells, early and mature B-lymphocytes and foetal brain cells	Kewett and Gilbert [21]
BA1	B-Lymphocytes, some granulocytic cells and neuroblastoma cells	Abramson *et al.* [22]
BA2	Some ALL and AML blasts and some neuroblastoma cells	Ash *et al.* [23]
Anti-Thy-1	Neuroblastoma, glioma, rhabdomyosarcoma and teratoma cells	Seeger *et al.* [24]
Anti-neurofilaments	Normal and tumour chromaffin cells	Debus *et al.* [25]

Table 2. Pattern of reactivity of MAbs to normal bone marrow cells and BM cells obtained from patients with leukaemia in remission

Antibody	Binding to normal BM cells	Binding to cells from leukaemic BM in remission
UJ13A	<1%	—
UJ167.11	—	—
UJ127.11	<1%	2%
A2B5	—	—
UJ181.4	<1%	<1%
M1N1	7%	10%
BA1	2%	3%
BA2	2%	2%
PI153/3	3%	6%
Anti-neurofilaments	—	—
Anti-Thy-1	1%	—

histological and cytological examination, similar to normal haemopoietic progenitors [13, 14], new approaches to the detection of malignant cells in bone marrow have been sought [15, 16].

With the binding of rabbit antisera to neurone-specific enolase and a panel of monoclonal antibodies, high levels of tumour cell detection have been claimed: one tumour cell in 10^4 to 10^5 normal cells, when normal bone marrow was artificially contaminated with neuroblastoma cells [12, 17]. However, this limit of detection and its clinical severity remain to be proven in bone marrow samples from patients treated for neuroblastoma.

Using a large panel of MAbs we could detect the remainder of the neuroblastoma cells in 11 patients and were able to define their membrane marker expression in order to investigate whether the presence of such cells precedes clinical relapse. From our results, we came to the conclusion that it is possible to reduce the initially applied panel of 11 antibodies to three antibodies: UJ 167.11, A2B5 and UJ 13A. The above mentioned antibodies are highly specific and therefore enable the detection of even a small number of NB cells. The fact that 10 patients developed a diffuse bone marrow involvement, in a median follow-up time of 1.5 years, provides evidence that these cells could be responsible for the relapse.

With respect to the limited number of cases studied, it seems that residual neuroblastoma cells reveal a certain unique pattern of surface antigens. This observation might be of prognostic significance

Table 3. Clinical features and immunocytochemical detection of residual neuroblastoma cells and clinical outcome

Patient No.	Age/sex	Therapy	Response	Neuroblastoma cell detection	Clinical outcome
1	2/M	DDP/CPM/VM26, 3 courses	PR	3%	BM relapse
2	4/F	ADR/CPM/VCR DTIC IFO/VP16, 10 courses	PR	1%	BM relapse
3	5/M	CPM/VCR/DTIC IFO/VP16, 11 courses, surgery	SD	4%	BM relapse
4	3/M	CPM/VCR/DTIC DDP/CPM/VM26, 8 courses	PR	0.1%	BM relapse
5	14 month/F	ADR/CPM/VCR/DTIC DDP/CPM/VM26, 4 courses	CR	3%	BM relapse
6	4 month/F	CPM, 2 courses	CR	0%	Alive without disease
7	6/F	ADR/CPM/VCR/DTIC IFO/VP16 DDP/CPM/VM26, 10 courses, surgery	PR	2%	BM relapse
8	G/F	ADR/CPM/VCR/DTIC IFO/VP16/DDP/CPM/VM26, 10 courses	SD	5%	BM relapse
9	21/F	ADR/CPM/VCR/DTIC IFO/VP16/DDP/CPM/VM26, interferon, surgery	R	0%	Tumour progression
10	5/M	ADR/CPM/VCR/DTIC DDP/CPM/VM26, 8 courses, interferon	PR	0%	Alive without marrow relapse
11	16/F	ADR/CPM/VCR/DTIC DDP/CPM/VM26, 10 courses, surgery	PR	0%	Tumour progression
12	9/M	ADR/CPM/VCR/DTIC IFO/VP16 DDP/CPM/VM26, 10 courses, interferon	PR	0.4%	Tumour progression
13	5 month/F	CPM, 2 courses	CR	%	Alive without disease
14	14 month/F	ADR/CPM/VCR DDP/VM26, 4 courses	PR	0.1%	Alive without marrow relapse
15	4/M	ADR/CPM/VCR/DTIC IFO/VP16 DDP/CPM/VM26, 4 courses	PR	1%	Alive without marrow relapse
16	3/F	ADR/CPM/VCR/DTIC IFO/VP16 DDP/CPM/VM26, 10 courses, surgery	PR	3%	Alive without marrow relapse

Abbreviations: VCR = vincristine; ADR = Adriamycin®; SD = stable disease; BM = bone marrow; CPM = cyclophosphamide DTIC = dacarbazine; CR = complete remission; CDDP = cisplatin; PR = partial remission; R = relapse.

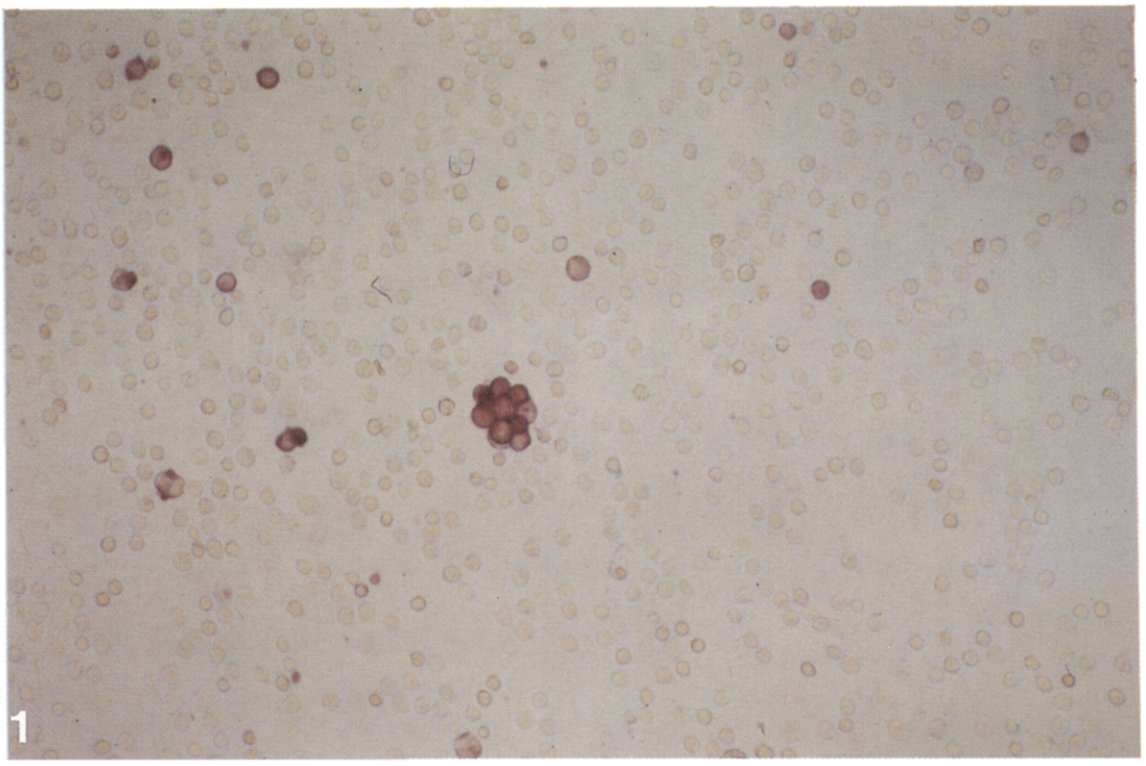

Fig. 1. Positive reaction for A2B5. Cells gathered in clumps stained red as well as a few single cells.

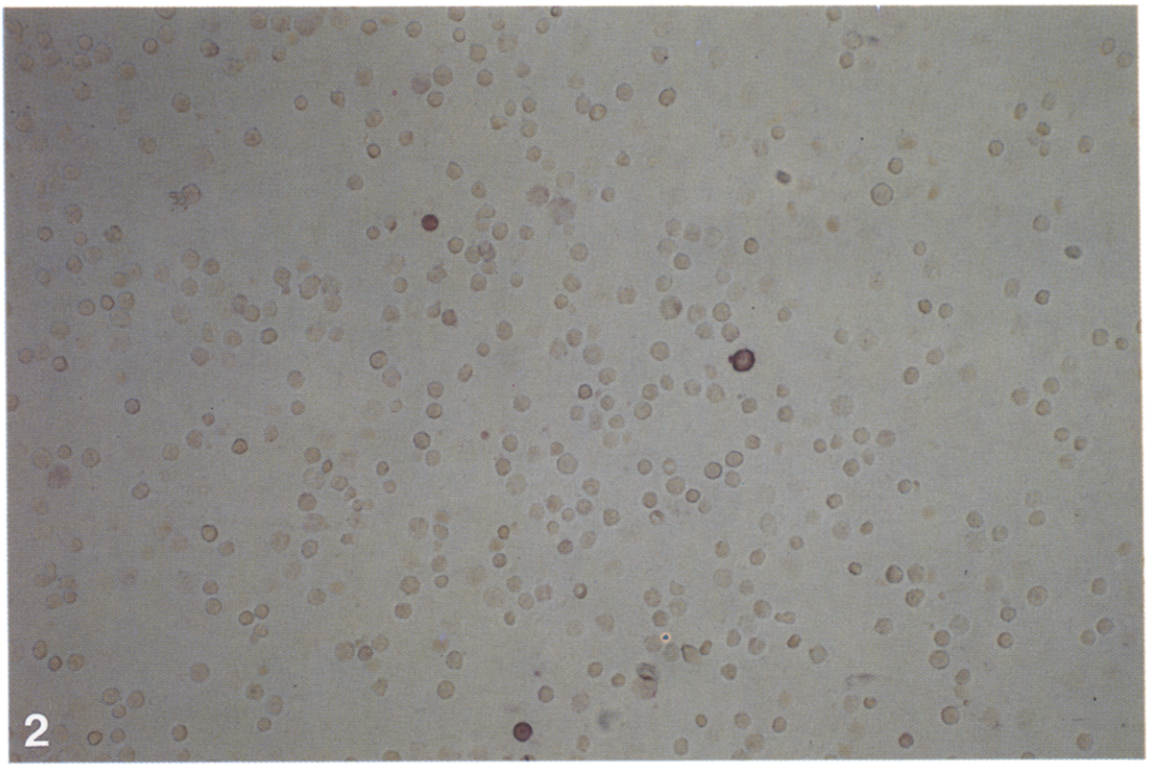

Fig. 2. Three single cells reveal positive reaction with the MAb UJ 13A.

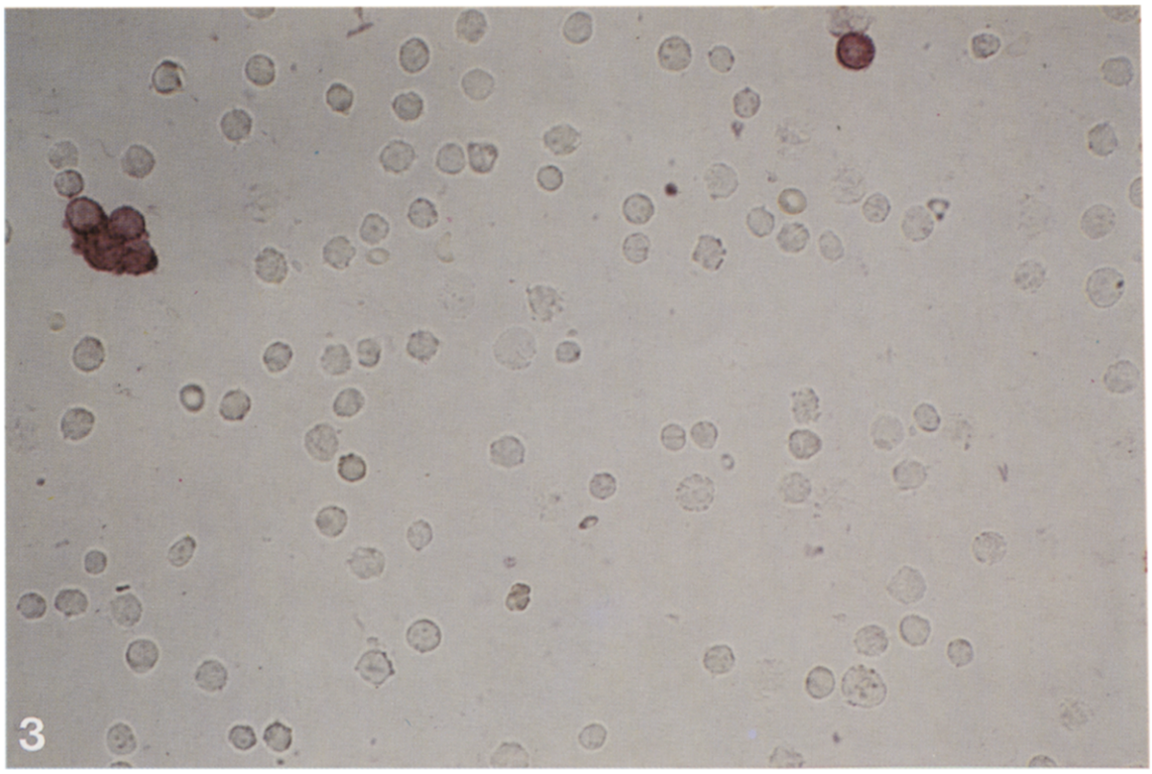

Fig. 3. One single cell and a small clump react with the marker UJ 167.11.

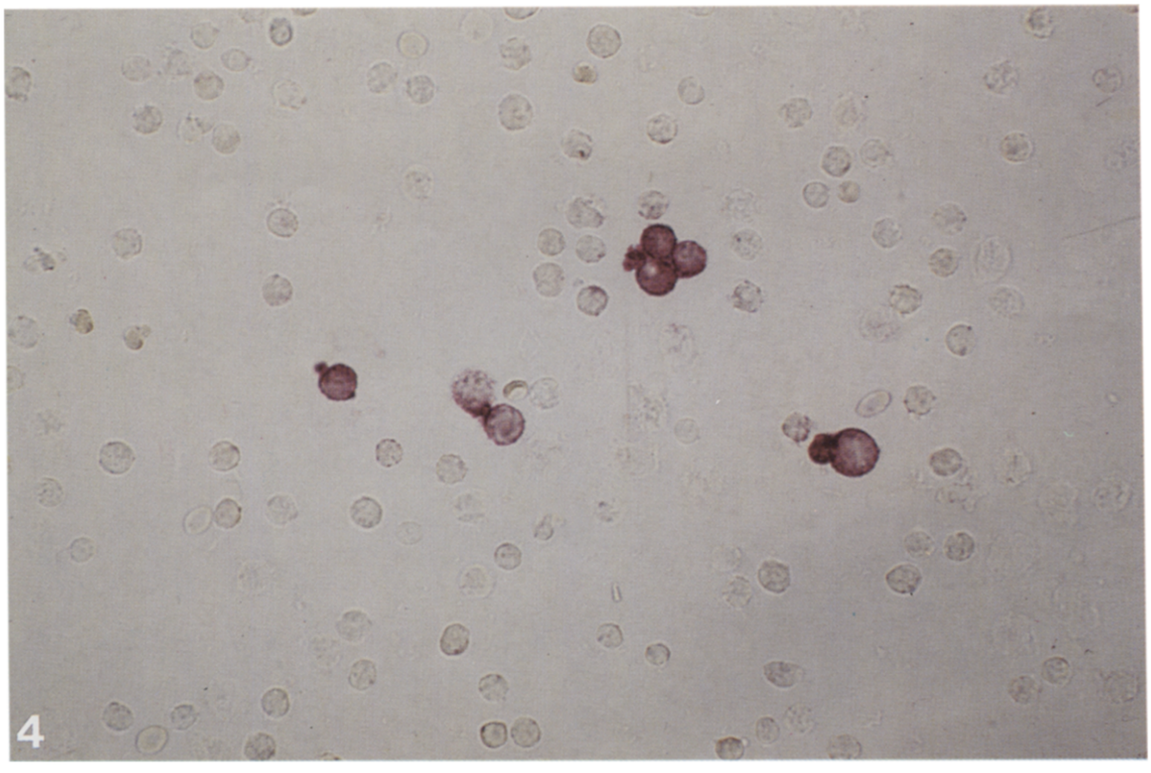

Fig. 4. Positive reaction for the marker Pl 153/3 *(patient No.* 2)

Table 4. Comparison between conventional investigations and immunological studies

Patient No.	Aspirates	Biopsies	Immunocytological analysis
1	—	+	+
2	—	—	+
3	+	+	+
4	—	—	+
5	+	+	+
6	—	—	—
7	—	—	+
8	+	+	+
9	—	—	—
10	—	—	—
11	—	—	—
12	—	—	+
13	—	—	—
14	—	—	+
15	—	+	+
16	—	+	+

Abreviations: — no neuroblastoma cell detected; + neuroblastoma cell detected.

Table 5. Phenotype of residual neuroblastoma cells

Patient No.	Monoclonal antibodies strongly reacting with residual neuroblastoma cells
1	UJ167.11 A2B5 UJ13A
2	A2B5 UJ167.11 PI153/3
3	UJ167.11 A2B5
4	A2B5 UJ13A UJ167.11
5	UJ167.11 A2B5
7	UJ167.11 A2B5 UJ13A
8	UJ167.11 A2B5 UJ13A
12	UJ167.11 A2B5 UJ13A
14	A2B5 UJ167.11
15	A2B5 UJ13A, UJ167.11
16	A2B5 UJ176.11

in so far as it reflects a selection of a clone resistant to chemotherapy. Moreover, this evidence suggests that bone marrow from every neuroblastoma patient, in remission or not, should be assayed immunologically in an attempt to identify small numbers of residual neuroblastoma cells.

Follow-up of these cases may provide a basis for the selection of subgroups of patients who would benefit from more intensive or alternative forms of chemotherapy before a full-blown relapse occurs.

Immunological phenotyping should also become an essential tool in testing 'purged' or 'cleansed' autologous bone marrow before transplantation, to make sure that 'contaminating' neuroblastoma cells are not returned to the patient. Because the APAAP method is easily established in any laboratory [18], it should facilitate deeper study on identifying neuroblastoma minimal residual disease phenotype in a large population. Finally, it must be noted that immunocytological studies cannot be evaluated in isolation from conventional cytohistological data, but it should be complementary to conventional analysis for a better definition of small tumour cell numbers in BM aspirates.

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Table 6. Comparison of initial neuroblastoma cell phenotype to phenotype of minimal residual disease

Patient No.	Phenotype of NB cells at time of diagnosis	Phenotype of residual NB cells
1	Anti-Thy-1, BA1, PI 153/3, UJ181.4, UJ167.11, UJ127.11, A2B5, UJ13A	UJ167.11 A2B5 UJ13A
4	Anti-neurofilaments, anti-Thy-1, UJ181.4, UJ167.11, UJ127.11, M1N1, A2B5, UJ13A, BA1, BA2	A2B5 UJ13A UJ167.11
5	Anti-Thy-1, UJ181.4, A2B5, M1N1, UJ167.11, UJ127.11, BA1, BA2	UJ167.11 A2B5
12	PI 153/3, UJ167.11, UJ127.11, anti-neurofilaments, BA1, BA2, A2B5, UJ181.4, UJ13A	UJ167.11 A2B5 UJ13A
14	A2B5, UJ167.11, UJ181.4, BA1, anti-Thy-1	A2B5 UJ167.11
15	A2B5, UJ13A, UJ167.11, BA1, BA2, PI 53/3	A2B5, UJ13A UJ167.11
16	UJ13A, A2B5, UJ127.11, anti-neurofilaments, BA1	A2B5 UJ167.11

Abbreviation: NB = neuroblastoma.

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