Immunological detection of neuroblastoma cells in bone marrow harvested for autologous transplantation

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Summary In about 50% of patients with stage IV neuroblastoma, micrometastases are present in the bone marrow when it is harvested for an autograft to follow induction therapy, and the risk of graft contamination by neuroblastoma cells has been the rationale for the use of a purging procedure. However, bone marrow metastases are detected with trephine biopsies which only explore the sites biopsied and do not reflect potential contamination of the pooled marrow harvested for autograft. A two-colour fluorochrome labelling method is described which permits as few as 1 neuroblastoma cell in 100,000 normal bone marrow cells from the autograft to be detected. Three monoclonal antibodies (UJ13A, H11 and 11.14) which react with neuroblastoma cells are used as single reagent in combination with a fourth anti-panleucocyte antibody. This method requires only 2h for the analysis of million normal marrow cells from the autograft, and is more effective than alkaline phosphatase staining with the same monoclonal antibodies. Results were compared with conventional techniques (four biopsies and four aspirates) carried out at the same time in 34 consecutive patients. Of 18 cases with negative aspirates and biopsies, neuroblastoma cells were detected in two autografts by the immunological method. Of 16 cases with positive aspirates and/or biopsies, 10 autografts were positive by the immunological method and six were negative. Trephine micrometastases were detected in 16 of the 34 patients, but the autograft contained malignant cells in only 12 of these patients and the immunological analysis demonstrated that the use of a purging procedure allowed the elimination of neuroblastoma cells from the autograft before its reinfusion to the patients.

Intensive chemotherapy followed by autologous bone marrow transplantation (ABMT) is now widely used as early consolidation therapy for stage IV neuroblastoma in children over one year of age (August et al., 1984; Philip et al., 1987; Graham-Pole et al., 1984; Pritchard et al., 1982; Hartmann et al., 1987; D’Angio et al., 1985). After induction therapy, at least 50% of such children fail to achieve complete remission and receive their bone marrow graft in partial remission. In these patients, scanty neuroblastoma cells may be detected in the marrow by the analysis of multiple biopsies although aspirates were often negative (Favrot et al., 1986; Favrot & Hervé, 1987; Franklin & Pritchard, 1983; Borstrom et al., 1985); this may be due to lack of sensitivity of cytological detection methods or to the fact that these clumps of neuroblastoma cells are not sucked out. One of the major issues in the treatment of neuroblastoma is thus to know whether bone marrow harvested for an autograft contains malignant cells or not, and whether these cells can be eliminated by an in vitro purging procedure before the graft is reinfused. It is therefore of major importance to develop accurate methods to detect and quantify rare neuroblasts in the marrow to be used for autograft. Neuroblasts have very low clonogenic efficiency in culture, however; cytogenetic analysis is not useful in detecting very small numbers of neuroblasts, and molecular biology techniques do not yet permit detection of less than 2% malignant cells (Favrot & Hervé, 1987). In theory, immunological analysis should be the optimal method of detection since monoclonal antibodies which recognise neuroblasts are now available (Allan et al., 1983; Kemsheda et al., 1983; Combaret et al., 1988; Favrot et al., 1988; Cheung et al., 1986; Evans et al., 1984). In practice, however, there remain technical problems due to the fact that anti-neuroblastoma MoAbs may stain a few normal marrow cells non-specifically, especially when the marrow is analysed after a course of chemotherapy. Immuno-cytotoxic methods (e.g. alkaline phosphatase or peroxidase immunostaining) preserve the cytological features of the cells and allow positively stained normal cells to be distinguished from malignant cells, but the method is time-consuming and the number of cells that can be analysed is limited. In this paper we describe a simple and brief method of two-colour fluorochrome labelling in which three monoclonal antibodies (UJ13A, H11 and 11.14) reacting with neuroblasts are used as single reagent in combination with an anti-panleucocyte antibody directed against normal marrow cells. This double marker analysis allows detection of as few as $10^{-4}$ malignant cells in the normal marrow. In this study and for 34 patients, the BM harvested for an autograft has been analysed with the immunological method and the results have been compared to those of the cytohistological analysis of four trephine biopsies and four aspirates. The same method has been used to demonstrate the elimination of the detectable residual malignant cells by the purging procedure.

Materials and methods

Patients and materials

Subjects were either unselected stage IV neuroblastoma patients over one year of age, treated in consolidation by our current LMCE (Lyon, Marseille, Curie, East of France Cooperative Group) protocol of high dose chemotherapy and ABMT (Philip et al., 1987a, b), or patients referred from other centres for inclusion in the Centre Léon Bérard pilot study of double ABMT (Philip et al., 1988). In the first group marrow was harvested after four to seven courses of induction therapy as previously described (Philip et al., 1987a). In the second group, patients usually received two courses of VP16 and CDDP before marrow harvesting (Philip et al., 1987b, 1988).

Morphological examination of the marrow was performed as previously described (Favrot et al., 1986). At the time of marrow harvesting under general anaesthesia, four marrow biopsies and four aspirates were performed in posterior and anterior iliac crests. Formalin-fixed trephines were analysed with conventional haematoxylin phloxin safran staining; two
spread films from each marrow aspirate were stained by May Grumwald Giemsa.

Harvested marrow was collected on citrate phosphate decahydrate mononuclear marrow cells were obtained by Ficoll separation on a blood cell processor (COBE 2991). Five million mononuclear marrow cells were taken for immunological analysis before purging, and the harvested marrow was then purified by an immunomagnetic depletion (IMD) procedure using five monoclonal antibodies (i.e. UJ13A, H-11, UJ127-11, UJ181-4 and xThy 1), as previously described (Favrot et al., 1987; Combaret et al., 1988; Trealeaven et al., 1984). A further five million mononuclear marrow cells were then taken for immunological analysis after purging.

**Normal bone marrow samples**

After informed consent, according to the Centre Léon Bérard ethical rules, marrow samples obtained under general anaesthesia from non-cancer patients or regenerating marrow samples from patients with neuroblastoma or malignant lymphoma in complete remission were used as control for the immunological analysis (see below).

**Immunological analysis**

UJ13A and H11 MoAbs (kindly provided by J. Kemshhead) and S-L 11.14 (kindly provided by J.C. Laurent) recognise antigens expressed by cells of neuroectodermal origin (11-14). These IgG antibodies strongly react with 90% of our patients’ tumour samples (either neuroblastoma cells in heavily involved marrow, at diagnosis or relapse, or primary tumours taken at surgery). The UJ13A and H111 MoAbs are included in the cocktail used for the purging procedure whereas 11.14 was selected in such study as a third marker which does not interfere with the purging cocktail. NKH1, (Couleur, France) is an IgM reagent claimed to be relatively specific for NK cells (Herend et al., 1983). An IgM anti-

**Two-colour fluorochrome immunostaining** Three samples (1 x 10⁶ marrow cells per sample, in suspension in 100 µl phosphate buffer saline (PBS) with 0.1% NaN₃) were incubated with the three anti-neuroblastoma monoclonal antibodies (one for each sample) in combination with either the anti-panleucocyte or NKH1. After 10 min at 24°C, samples are washed once in PBS and incubated with TRITC anti-mouse IgG specific and FITC anti-mouse IgG specific (Southern Biotechnology Associates, ref. 1020 and 1030) for 10 min at 24°C (specificity of the class-specific antisera has previously been checked on monoclonal antibodies of different subclasses). Samples are then washed twice, maintained in PBS-glycerol and analysed in a fluorescent Zeiss microscope with a 40:1 objective, a 490 nm excitation filter and a K530 barrier filter. Negative controls include one sample stained with the second layers without monoclonal antibody.

**Alkaline phosphatase immunostaining** Marrow cells in suspension at 6 x 10⁶ ml⁻¹ in PBS were cytocentrifuged into glass (100 µl of sample at 70 g for 5 min in a Shandon cytospin) (Warnke et al., 1983; Maritaz et al., 1988). Immunochemical staining is performed using an indirect three-stage immunoenzymatic procedure (20, 21) with alkaline phosphatase (Dakopatts, Copenhagen, Denmark). Briefly, six air-dried slides are fixed for 5 min with acetone at 4°C, incubated for 60 min with MoAbs (three with UJ13A and three with 11.14) then for 30 min with enzyme-conjugated rabbit anti-mouse immunoglobulins (Dakopatts) and for 30 min with enzyme-conjugated swine anti-rabbit immunoglobulins (Dakopatts). Washes are done with Tris buffer. The final step consists of a 15-min incubation with Naphthol-As-Mx phosphate, dimethylformamide, levamisole and fast red (Sigma Co., St Louis, USA). Slides are counterstained with haematoxylin, mounted permanently with glycerin and evaluated under an optical microscope. Negative controls with non-specific antibody and monoclonal antibodies with MoAbs recognising class I antigen on normal marrow cells are included in each test. Slides were considered technically unsatisfactory and were not evaluated if any of the following was observed: (1) positive staining in the negative control; (2) high background staining in the test samples; (3) disrupted morphology with absence of recognisable cellular structures.

**Limit of detection** The methods were shown to enable the detection of as few as 10⁻⁵ neuroblastoma cells in the marrow if 3 x 10⁹ cells are analysed in two-colour fluorochrome labelling and six smears in alkaline phosphatase (blind study) (Maritaz et al., 1988).

**Results**

**Reactivity of 11.14, UJ13A and H.11 with normal haematopoietic cells**

Ten marrow samples from healthy donors were analysed by the two-colour fluorochrome labelling; all three MoAbs, used as single reagent, stained less than 1% haematopoietic cells. In patients treated for malignant lymphoma and in complete remission, samples of regenerating marrow taken after chemotherapy contained up to 10% marrow cells stained with one or the other reagent. Such non-specific staining was also observed in our neuroblastoma patients in complete remission. Two-colour fluorochrome labelling with an anti-

**Comparison of the morphological detection of bone marrow micrometastasis with the immunological detection of malignant cells in the autograft:** 34 cases

Samples for morphological analysis of the marrow and immunological analysis of the autograft were taken on the same day during the harvest surgical procedure. Four biopsies and eight spread films (two per aspiration site) were analysed for each patient. Three million cells were analysed in two-colour fluorochrome labelling and six smears (6 x 10⁹ cells per smear) were analysed by alkaline phosphatase immunostaining before and after the purging procedure. (see Table I).

In 16 cases, both morphological examination of the marrow and immunological analysis of harvested marrow cells were normal. In 18 cases, malignant cells were detected in the marrow by one or the other method as detailed below. In 3 cases, biopsies, aspirates and immunological analysis were positive; two to three of the eight morpho-

**In 12 cases, biopsies were positive but aspirates were negative:** three patients had only one positive biopsy but three patients had three to four positive biopsies with one or two detectable clumps in each. Before the purging procedure, the immunological analysis permitted to detect 10⁻³ to 10⁻⁵ residual neuroblastoma cells in the autograft of only six of these patients.

In one case, the four biopsies were negative but the aspirates contained rare atypical lymphoid-like malignant
cells; the immunological analysis of the autograft confirmed the presence of 15% malignant cells.

In two cases the four biopsies and four aspirates were completely negative, but clumps of malignant cells were detected in the autograft by the two-colour fluorochrome labelling, the contamination being 1 NBL cell in 10^5 marrow cells.

Finally, when marrow was analysed after the purging procedure they did not contain any residual neuroblastoma cell detectable by immunological analysis.

The combination of these two immunological methods enables detection of one NBL cell in 10^5 marrow cells in the autograft. Particularly in 11 of the 12 cases of contaminated autografts, malignant cells were detectable by the two-colour fluorochrome labelling; alkaline phosphatase immunostaining was positive in only five cases, one only with negative immunofluorescence and four with concomitant positive immunofluorescence.

**Discussion**

In this series of 34 consecutive neuroblastoma patients entered in an AMBT programme, 16 had marrow micro-metastases detectable by cytohistological examination of four trephine biopsies and four aspirates on the day of marrow harvest; in agreement with our previous results, biopsies were more accurate than aspirates in detecting these rare neuroblasts. The marrow infiltration by neuroblastoma cells is very focal and biopsies or aspirates only explore four iliac sites whereas the marrow harvested for an autograft is taken from the entire iliac site. The immunological analysis of the cells from the whole harvested marrow enabled us to demonstrate that the autograft was contaminated by neuroblasts in 12 of the 34 cases analysed, 10 with concomitant positive biopsies and/or aspirates, and two with negative biopsies and aspirates. The autograft contamination could be quantified and ranged from 10^-2 to 10^-5 malignant cells; after *ex vivo* purging procedure, none of the autografts contained residual malignant cells detectable by immunological analysis; less than 10^-3 neuroblasts were thus potentially left when the marrow was reinjected to the patient. In the seven cases in which one of the biopsies was positive and malignant cells were undetectable in the harvested marrow by immunological analysis, the autograft contained less than 10^-5 NBL cells or could even be normal if very focal clumps of malignant cells failed to be aspirated during the harvesting procedure.

The great sensitivity of the immunological detection is due, first, to the Ficoll separation of the mononuclear cell population before the analysis. Separation of the marrow cells, either on a Ficoll gradient or on a discontinuous sedimentation gradient, had been reported to enrich the marrow population with malignant cells, by eliminating red cells and granulocytes, and to improve the examination (Maritaz et al., 1988; Bayle et al., 1985; Hunter et al., 1987). The sensitivity of the method described here is due, secondly, to the objective characterisation of malignant cells. The two-colour fluorochrome labelling method enables an objective distinction to be made between the few lymphocytes which stain with both the anti-panleucocyte and the anti-neuroblastoma antibodies, and isolated neuroblastoma cells which are panleucocyte negative. Similarly, the immunohistological staining permits identification of pseudolymphoid neuroblasts by their membrane positivity. Finally, the number of cells analysed for each case in this study (3 x 10^4 in two-colour fluorochrome labelling and 6 x 10^5 in alkaline phosphatase) allows detection down to 10^-3 neuroblasts by immunological analysis. In this context, two-colour fluorochrome labelling offers several advantages when compared to alkaline phosphatase staining. In the first method, the processing of 3 x 10^4 mononuclear cells only requires 90 min once the marrow has been harvested and their analysis on three different slides takes 30 min. Immunostaining and analysis of six smears by the alkaline phosphatase method usually takes 4-5 h and consequently limits the total number of mononuclear cells to be analysed. The greater sensitivity of two-colour fluorochrome labelling is due to the larger number of cells analysed. Two-colour fluorochrome labelling thus appears, both for its sensitivity and its simplicity, as an optimal method to develop routinely in a laboratory involved in clinical programmes in ABMT. The method can be easily adjusted to the analysis of the marrow in various solid tumours. In small cell lung cancer, the three markers used in this study (UJ13A, H11 and 11.14) strongly react with the malignant cells and the method of detection is strictly similar; in other tumours such as breast cancer, relevant markers of the malignant cells are available.

For clinicians, such an analysis clearly selects a group of patients whose autograft is contaminated with malignant cells and for whom the use of a purging procedure is largely justified. In addition, the immunological analysis enables the efficiency of clinical purging procedures to be checked and allows the elimination of malignant cells to be quantified. Such data are of particular interest in a disease such as neuroblastoma in which two patients out of three never reach complete remission and are thus autografted in partial remission. To determine whether relapses are due to the failure of high dose chemotherapy or to the rejection of malignant cells is a prerequisite in the improvement or modification of therapeutic protocols.

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