Immunohistochemical detection of neuroblastoma in frozen sections of bone marrow trephine biopsy specimens

M M Reid, A J Malcolm, A G McGuckin

Abstract

Frozen sections from bone marrow trephine biopsy specimens from children with disseminated neuroblastoma were stained using the monoclonal antibody UJ13A. An immunoalkaline phosphatase technique was the preferred staining method. Acceptable histological detail was obtained from this material and deposits of tumour cells detected. Some apparently fibrous tissue was also stained by this antibody. The results show that this immunohistological approach is feasible and provide encouragement for its addition to the range of investigations currently available for assessing the marrows of children with this disease.

When assessing children with disseminated neuroblastoma, histological examination of bone marrow trephine biopsy specimens is more rewarding than either standard cytological or immunocytochemical methods of examining aspirated material.¹ The interpretation of conventional histological preparations, however, remains uncertain in many cases.² The ability to detect neuroectodermal antigens in the marrow cores of such patients may improve interpretation of their specimens. This report describes an immunohistological technique using frozen sections of marrow cores stained with the monoclonal antibody UJ13A and shows that such an approach is feasible.

Methods

Fresh iliac crest trephine biopsy specimens, taken with a paediatric Jamshidi needle as part of routine staging or re-evaluation, were divided into two portions. One was processed for conventional histological examination, the other embedded in a glycerol based compound (OCT, Miles Scientific), rapidly frozen using isopentane cooled in liquid nitrogen, then stored at -70°C until required. The biopsy specimens studied were obtained from four patients with disseminated neuroblastoma at diagnosis two to four months after starting treatment. All patients were known to have UJ13A positive tumour cells.

SECTIONING AND FIXATION

Sections, $7\text{ }\mu\text{m}$ thick, were cut using a cryostat (Reichert-Jung Frigocut) at a temperature between -15 and -25°C . A variety of section

adhesives was tested, including aminopropyltriethoxysilane (APES), poly-L-lysine, and chrome alum gelatin. This last was found to be most useful in our hands and was used subsequently at all times. It was particularly necessary to ensure adherence of sections of hypoplastic marrow, with relatively normal architecture, which proved the most fragile. Sections were dried overnight at room temperature, fixed in acetone for 10 minutes, air dried and washed in TRIS buffered saline (TBS), pH 7.6, for two consecutive five minute washes, followed by incubation in non-immune rabbit serum for 10 minutes.

IMMUNOSTAINING

Early experiments compared the indirect immunoperoxidase with the comparable immunoalkaline phosphatase technique. In the immunoperoxidase technique heavy haemosiderin deposits in many specimens appeared brown and could be confused with positively stained cells. Native peroxidase activity of haemopoietic cells was not always easily overcome. The ability to block endogenous alkaline phosphatase activity increased contrast. The authors also preferred the colour reaction product in the immunoalkaline phosphatase method. Immunoalkaline phosphatase was therefore the preferred option.

The primary antibody was UJ13A (kindly donated by Dr J Kemshead, Tumour Immunology Laboratory, Institute of Child Health). It is a murine monoclonal antibody raised against fetal brain and recognises the cell adhesion molecule N-CAM (personal communication, Dr J Kemshead) present on neuroectodermal cells. It was diluted with non-immune rabbit serum in TBS. Optimal working dilutions were established for each batch of antibody.

Secondary antibody was an alkaline phosphatase conjugated rabbit anti-mouse antibody (Dako, No D314). It was used at a final working dilution of 1/10 in 20% non-immune rabbit serum in TBS.

Sections were incubated with UJ13A for 30 minutes at room temperature in a humid environment. A test section was simultaneously incubated with rabbit serum omitting primary antisera, which acted as a negative control. A section from a primary tumour biopsy specimen was used as a positive control. Sections were washed for 10 minutes in TBS. The secondary antibody was applied similarly and washed in TBS. Alkaline phosphatase was visualised using naphthol AS-TR phosphate in

Department of
Haematology, Royal
Victoria Infirmary,
Newcastle upon Tyne
A J Malcolm
A G McGuckin
M M Reid

Department of
Pathology, University
of Newcastle upon
Tyne
M M Reid

Correspondence to:
Dr M M Reid, Department
of Haematology, Royal
Victoria Infirmary,
Newcastle upon Tyne NE1
4LP

Accepted for publication
8 November 1989

Figure 1 Positive tumour deposit after treatment. Note strongly positive eosinophils and faint background positivity. (Immunoperoxidase.)

a standard cytochemical technique. Endogenous alkaline phosphatase activity was blocked with levamisole incorporated into the enzyme development medium. Sections were counterstained with Mayer's haematoxylin, mounted in an aqueous mountant, and viewed with a standard light microscope.

Results

Although the preferred immunostaining technique was immunoalkaline phosphatase, an example of successful immunoperoxidase staining is shown in fig 1. Acceptable histological detail is essential for this technique to be worth pursuing. Examples of variable cellularity and architecture are shown in figs 2 to 4.

Figure 2 shows UJ13A positive tumour cells, normal marrow cells, and at least one deposit of UJ13A negative tumour in a biopsy specimen taken four months after starting aggressive chemotherapy. Similar histological appear-

Figure 2 Positive tumour cells, normal haemopoietic cells and negative tumour cells. (Immunoalkaline phosphatase.)

ances had been found in the conventionally handled biopsy material. Figure 3 shows multiple small deposits of residual tumour in hypoplastic marrow two months after starting treatment. Figure 4 shows UJ13A positive tissue in streaming whorls of apparently fibrous material in marrow from an intensively treated patient. Within it are some cells with large, oval, or elongated nuclei whose identity is unclear. Similar appearances can be found at diagnosis (data not shown). Figure 5 shows a number of UJ13A positive osteoblasts along the edge of growing bone. Golden coloured deposits of haemosiderin are shown in figs 3 and 4, clearly contrasting with the red reaction product of the immunoalkaline phosphatase technique.

Discussion

Formalin fixed, paraffin wax embedded trephine biopsy specimens from children with neuroblastoma can be examined by immunohistochemical techniques using, for example, antisera against neurone specific enolase.³ Formalin fixation and decalcification procedures can mask or even destroy many cell antigens, thus restricting the range of investigations which can be carried out. UJ13A reacts strongly with most neuroblastomas, but the antigen or epitope which it recognises is rendered undetectable by formalin.⁴ Frozen sections or some alternative fixative⁵ are required for its use in histological sections.

Frozen, undecalcified bone marrow is a notoriously difficult tissue from which to obtain untraumatised sections which adhere well to the slide. Hypoplastic marrow with relatively normal architecture provided the greatest challenge. Nevertheless, with application and the readiness to persevere, acceptable histological detail can be obtained, examples of which are contained in this report. The labour intensive nature of the work, however, when compared with handling paraffin wax embedded, decalcified material, is a drawback.

The high level of non-specific peroxidase activity in haemopoietic cells and the preference by the authors for the colour reaction in the immunoalkaline phosphatase technique were the major factors affecting the choice of immunostaining technique. Only simple, two stage immunoperoxidase and immunoalkaline phosphatase techniques were

Figure 3 Hypoplastic marrow with several small deposits of tumour cells after treatment. (Immunoalkaline phosphatase.)

Figure 4 Positive material within "fibrous" tissue, after treatment. (Immunoalkaline phosphatase.)

studied. The more complex peroxidase-anti-peroxidase (PAP) or alkaline phosphatase-anti-alkaline phosphatase (APAAP) techniques might have given both a stronger visual signal and reduced non-specific background staining.

UJ13A stained frankly primitive cells in marrow at diagnosis (data not shown). Infiltration at diagnosis is usually easy to detect, and conventional histological, cytological, and immunocytochemical stains are much simpler to perform. The use of this antibody in frozen sections, however, draws attention to small tumour deposits in hypocellular or fibrotic marrow after treatment has been started and also shows that antigen negative tumour cells

Figure 5 Positive osteoblasts. (Immunoalkaline phosphatase.)

may coexist with positive cells in the same biopsy specimen. The presence of immunopositive material in hypocellular "fibrous" tissue both at diagnosis (data not shown) and, in some cases, after treatment, is worth noting. This material may be akin to that found in more differentiated or partially treated primary neuroblastomas. It is possible that some of the "fibrous" material detected by conventional histological techniques is a product of neuroectodermal cells rather than reactive bone marrow fibroblasts.

Osteoblasts often stained with UJ13A, confirming previous observations by us¹ and others,⁵ but their individual morphological appearances and restriction to the edge of bone trabeculae usually makes their distinction from tumour simple. This contrasts with the potential problem of interpreting immunofluorescence studies of cell suspensions. Osteoblasts are easily aspirated from the marrow of children, often as groups or clumps, and in intensively treated children may constitute a substantial proportion of aspirated cells.

In summary, this report shows that, despite the technical difficulties, it is possible to obtain useful information from immunohistochemical studies of frozen bone marrow in this disease. These techniques could be extended to other monoclonal antibodies. Comparison of a range of immunohistochemical techniques using frozen and formalin fixed material with conventional histological examination in a large series of children may lead to a clearer picture of the importance of the various histological abnormalities of the marrow in children with disseminated neuroblastoma.

A G McGuckin is supported by a grant from the North of England Children's Cancer Research Fund.

- 1 Carey PJ, Thomas L, Buckle G, Reid MM. Immunocytochemical examination of bone marrow in disseminated neuroblastoma. *J Clin Pathol* 1990;43:9-12.
- 2 Reid MM, Hamilton PJ. Histology of neuroblastoma involving bone marrow: the problem of detecting residual tumour after initiation of chemotherapy. *Br J Haematol* 1988;40:487-90.
- 3 Ironside JW, Shiach CR, Cuthbert AC, Robinson EA. Histology of neuroblastoma involving bone marrow. *Br J Haematol* 1989;71:565-6.
- 4 Carey PJ, Malcolm AJ, Pearson ADJ, Kemshead JT, Reid MM. Immunocytochemical detection of tumours of neuroectodermal origin. *J Clin Pathol* 1988;41:586-9.
- 5 Oppedal BR, Storm Mathisen I, Kemshead JT, Brandtzaeg P. Bone marrow examination in neuroblastoma patients; a morphological immunocyto- and immunohistochemical study. *Hum Pathol* 1989;20:800-5.