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brain cell neuroblastoma

A Monoclonal Antibody Detecting an Antigen Shared by Neural and Granulocytic Cells

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Summary

A monoclonal antibody, MI/N1, is described that reacts predominantly with fresh neuroblastoma tissue, human neuroblastoma cell lines, and cells of the myeloid lineage. Investigation of the binding of this antibody to four different neuroblastoma cell lines showed CHP 100 bound approximately 4 times more antibody than CHP 126. Only 30% of the cells in the line CHP 100 bound MI/N1 as determined by indirect immunofluorescence. Thus, both quantitative and qualitative differences in the expression of antigen recognised by MI/N1 are detected on human neuroblastoma cell lines. Inasmuch as only five of eight marrow aspirates heavily infiltrated with neuroblasts bound the monoclonal, this also suggests a heterogeneity in antigenic expression on fresh tumour cells. Absorption studies indicate that the antigen recognised by MI/N1 is present on human foetal brain and adult human cerebellum. At a dilution of 1/750, equal volumes of foetal brain and adult cerebellum absorbed out 30 and 60% of the reactivity to the human neuroblastoma cell line CHP 100. No reactivity was found towards murine neuroblastoma cells or rat brain.

Expression of antigen on cells in the myeloid lineage appears dependent upon their stage of maturation, increasing as cells mature to neutrophils and eosinophils.

It is suggested that the quantitative and qualitative differences seen in the expression of antigen on neuroblastoma cells may relate to their being blocked at different stages of differentiation.

Although some childhood malignancies of neural crest origin have shown improvements in clinical response in the last decade this has not been the case for neuroblastoma (6, 17). Despite the development of new chemotherapeutic regimes, prognosis still remains poor in this malignancy, especially in older children (over the age of 2 years) presenting with stage 3 or 4 of the disease (2, 4).

An immunologic approach has been sought to aid the early detection of metastatic spread of neuroblastoma, especially with reference to infiltration of the bone marrow (1, 3). Present methods of identifying tumour infiltration rely on an histologic analysis of biopsy samples where it is often difficult to definitively distinguish low levels of neuroblasts from normal haematopoietic blast cells present in the bone marrow (12). Antibodies specific for neuroblastoma tumour cells or neural tissue would serve clearly to identify tumour cells and might in addition have a use in autologous bone marrow transplantation, *i.e.*, for removing residual tumour cells (23).

In the search for such monospecific reagents, somatic cell hybridisation has been used to produce monoclonal antibodies (18, 19). In this paper, we describe a new monoclonal antibody, raised against a neuroblastoma cell line, which reacts with neural tissue and at least one haematopoietic cell type.

MATERIALS AND METHODS

MICE AND IMMUNISATIONS

Female BALB/c mice, 2 to 3 months old, reared under specific pathogen-free conditions were immunised by intraperitoneal (IP) injection with 10^7 human neuroblastoma cells (line CHP 100) in complete Freund's adjuvant. Thirty days later, a secondary injection of 10^7 cells was given IP without adjuvant, and the spleens from these mice removed 5 days later for cell fusion (14).

CELL HYBRIDISATION AND ASSAY FOR ANTIBODY SECRETION

Spleen cells were fused with the mouse myeloma cell line P3-X63-Ag8-653 (11) using polyethylene glycol 1500, following the method of Köhler and Milstein (18, 19). Cells were plated out in 1.0 ml aliquots at 2×10^6 cells/ml in the presence of 5×10^4 peritoneal exudate cells from BALB/c mice. After the selection for hybrids in medium containing hypoxanthine, aminopterin, and thymidine (20), supernatants from hybrid cultures were assayed for antibody binding to the human neuroblastoma line CHP 100 (22).

Supernatants were incubated with 2×10^5 cells for 30 min at room temperature in Cooke microtitre plates that were prewashed in phosphate-buffered saline (PBS) containing 1% foetal calf serum (FCS). After washing 3 times with PBS/1% FCS, the cells were incubated for 30 min at room temperature with 30 ng of affinity purified ^{125}I-goat anti-mouse immunoglobulin (Ig) (7 μCi ^{125}I/μg protein). Iodinated antibody binding to a pellet of cells was determined in a LKB 1280 Ultragamma counter. All assays were in duplicate, and supernatants giving at least 2 times background were taken as positive. Cells from such wells were cloned 3 times by limiting dilution in the presence of 5×10^4 peritoneal exudate cells.

For ascites preparation, mice were primed with 200 μl of Pristaine oil 2 wk before immunisation with 5×10^6 hybrid cells (15). Ascites fluid was collected 2 to 3 wk later and ultracentrifuged before use.

CELL LINES

Human neuroblastoma cell lines CHP 100, 212, 134 and 126 (obtained from Dr. Schlessinger, Children's Hospital, Philadelphia) which were grown at 37°C in a 6% CO_2 incubator using Roswell Park Memorial Institute 1640 medium supplemented with 10% FCS, 2 mM glutamine, penicillin (100 IU/ml), and streptomycin (100 μg/ml). When approaching confluency, cells were split 1:3/1:5 using tryspin/ versene to remove adherent cells from the dishes when necessary.

Leukaemic cell lines growing in suspension culture were grown at 37°C in 10% CO_2 using Roswell Park Memorial Institute 1640 medium, 10% FCS, and the above supplements.

IMMUNOFLUORESCENCE

Indirect immunofluorescence using fluorescein-conjugated F(ab')₂ goat anti-mouse F(ab')₂ was carried out as described in Greaves and Janossy (7). Cells were examined using a Zeiss fluorescent microscope with incident illumination and by flow cytofluorimetry using a fluorescence activated cell sorter (FACS-I, Becton Dickinson, Mountain View, CA) (9).

PREPARATION OF FRESH NEURAL TISSUE

Within 24 hr of death, either foetal or adult brain suspensions were washed 3 times in PBS/1% FCS and centrifuged in a Beckmann microfuge (5 min at 10,000 × g). Two hundred μl of an ascites preparation of antibody were added to the pelleted neural material (200 μl packed volume), and the mixture was incubated at 4°C for 60 min.

After this period, the tubes were centrifuged at 10,000 × g for 5 min, and an assay for residual antibody in the supernatant was undertaken using CHP 100 cells and ^{125}I-goat anti-mouse.

RESULTS

CHARACTERISATION OF ANTIBODY

After fusion, cells were plated out into 50 wells (2 × 10⁶/well) and left for 14 days in the presence of medium containing hypoxanthine, aminopterin, and thymidine. At least one hybrid colony and often many more were seen in each well. Over the subsequent 2 wk, supernatants from the cultures were assayed for antibodies to the human neuroblastoma cell line CHP 100 by an indirect binding assay using ^{125}I-labeled goat anti-mouse immunoglobulin. In eight of 50 supernatants, antibodies to human neuroblastoma cells were detected, and cells from each of these cultures were cloned three times by limiting dilution to ensure monoclonality. One of these eight cell lines was found to secrete antibodies with restricted binding activity and was investigated further (Table 1).

REACTIVITY OF MI/NI WITH NEUROBLASTOMA CELL LINES

To determine if MI/N1 could be used as a general marker for human neuroblastoma cells, several lines were assayed for their ability to bind the antibody. Under conditions where the ^{125}I-anti-

mouse Ig was in excess, considerable variation in the binding of the monoclonal to different neuroblastoma cell lines was detected (Fig. 1). Maximal binding was found against the line CHP 100, and the shape of the titration curve indicates that to a dilution of ¹⁄₁₆₀ the monoclonal antibody was saturating all antigenic sites (Fig. 1). Only approximately 20 to 25% of the activity to CHP 100 was found upon titration of MI/N1 to saturation against the cell lines CHP 212 and 126. To determine if the spectrum of binding observed was due to either the reactivity of subpopulations occurring at different frequencies in the lines tested or to uniform quantitative differences, cells with MI/N1 antibody bound under saturating conditions were stained with fluorochrome-labeled anti-mouse Ig and analysed by both fluorescence microscopy and with the fluorescence-activated cell sorter (FACS).

By light microscopy, only approximately 30% of the cells in the line CHP 100 appeared fluorescent using either a culture supernatant or an ascites preparation of antibody. This heterogeneity was also seen upon FACS analysis of cells, suggesting either quantitative and/or qualitative differences in the expression of antigen occur within this population (Fig. 2). No morphologic differences could be distinguished between fluorescent positive and negative cells within the cell line, and antigenic expression did not significantly change when cells were either growing exponentially or approaching confluency. The other cell lines examined were homogeneous in their staining profiles being either weakly fluorescent positive on all cells or negative as determined by both microscopy and FACS analysis. Thus quantitative differences in antigenic expression seem to occur between the lines CHP 134, 126, and 212. Detection of the binding of MI/N1 to these latter neuroblastoma lines by either fluorescence or iodinated goat anti-mouse Ig appears to yield conflicting results with cells being positive for binding in a modified radioimmune assay and yet negative by fluroescence. This presumably reflects the relative sensitivity of the two assays.

The antigen recognised by MI/N1 would appear to show some species specificity because no binding to the mouse neuroblastoma line ICRF N.1. (a clone of C-1300) could be detected under conditions where strong binding to the human line CHP 100 was

Table 1. *Binding of MI/N1 to neural and haemopoietic cell lines*[1]

Line	Origin	Binding above background	
		Culture supernatant	Ascites
CHP 100	Neuroblastoma	×6	×10.3
UCH-P45	Astrocytoma		
NALM 1	Ph[1+] pre-B common acute lymphoblastic leukaemia (cALL)[2]		
KM3	Non-B, non-T cALL		
DAUDI	B lymphoma lacking HLA		
RAJI	B lymphoma		
HPB-TALL	T-ALL	×2.8	×3.6
HL 60	Promyelocytic leukaemia	×8.6	×19.3
K562	Ph[1+] myeloid leukaemia		

[1] Two × 10⁵ cells of each line were incubated with either (1) undiluted culture supernatant or (2) an ascites preparation of the antibody diluted 1/250, followed by 100,000 cpm of ^{125}I-goat anti-mouse Ig as described in "Materials and Methods." The ratio of counts bound to the cell line over a background (obtained by incubating cells with ^{125}I-goat anti-mouse Ig) is given above. All backgrounds were in the range of 200 to 350 cpm.
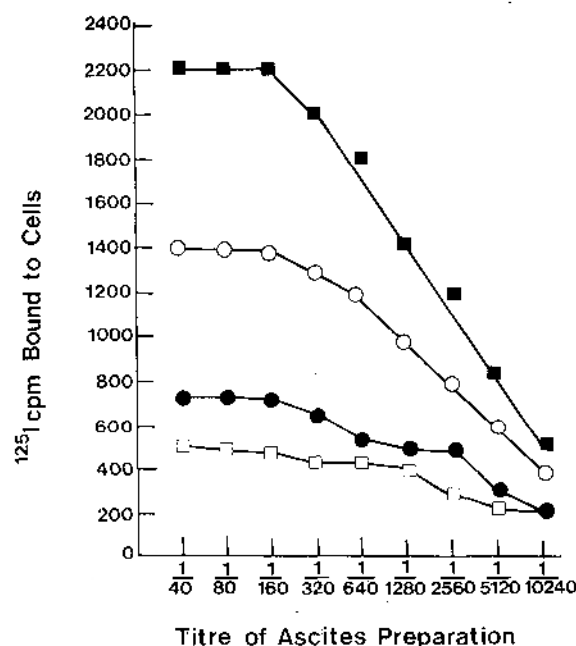
[2] Ph[1+], Philadelphia chromosome.

Fig. 1. The binding of monoclonal antibody MI/N1 to different human neuroblastoma cell lines. Two × 10⁵ cells of each line were incubated with differing dilutions of MI/N1 followed by 100,000 cpm of ^{125}I-goat anti-mouse as described in Materials and Methods. ■, binding to CHP 100; O, binding to CHP 134; ●, binding to CHP 212; □, binding to CHP 126.
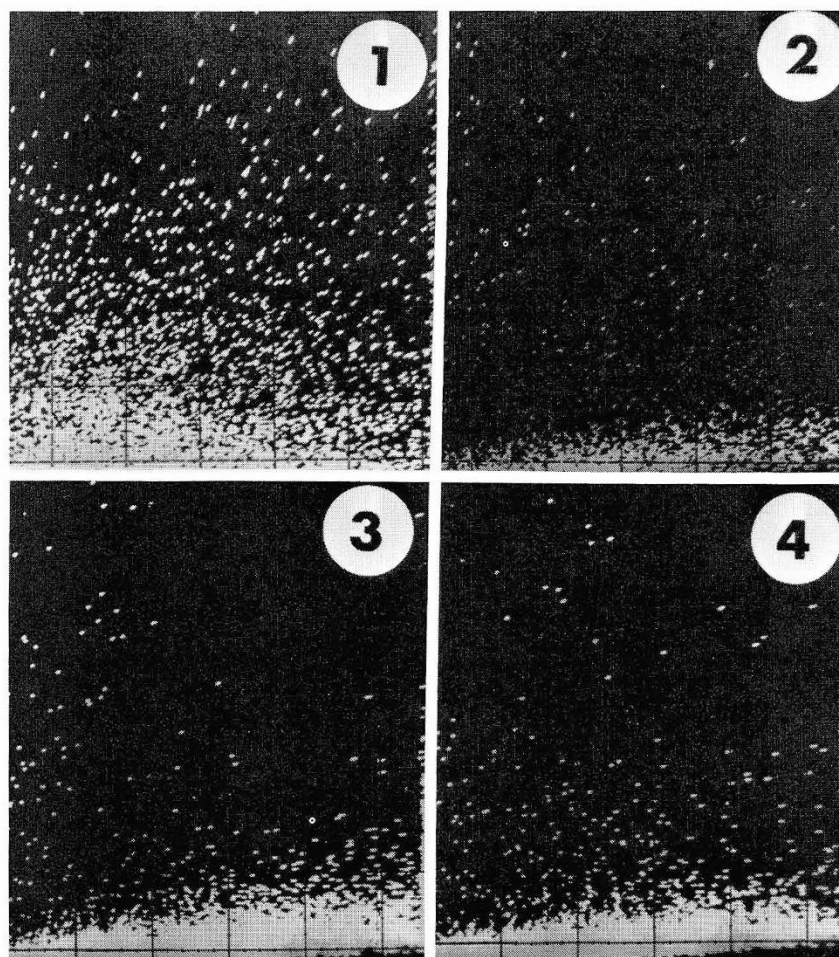
Fig. 2. Heterogeneity in the binding of MI/N1 to different human neuroblastoma cell lines as determined by FACS analysis. *Ordinate,* relative immunofluorescence intensity; *abscissa,* relative cell size. Cells (10^6) of each line (CHP 100 and CHP 212) were incubated with MI/N1 (1/100) for 30 min at 4°C and, following washing, for an equivalent time with fluroescein conjugated F(ab')₂ goat anti-mouse F(ab')₂. Cells were analysed for fluorescence using microscopy and the cell sorter and compared with control cultures incubated with the second antibody only. *1,* CHP 100 cell line incubated with MI/N1-FACS profile; by fluorescence microscopy 30% of cells were positive; *2,* CHP 100 cell line control culture-FACS profile; by fluorescent microscopy cells were all negative; *3,* CHP 212 cell line incubated with MI/N1-by fluorescent microscopy all cells were negative; *4,* CHP 212 cell line-control culture; by fluorescent microscopy all cells were negative.

found (Fig. 3). Furthermore, MI/N1 does not react with the human astrocytoma line UCH-P45, suggesting that the antigen is restricted to neuronal rather than neuroglial cells (Table 1).

REACTIVITY WITH FRESH NEURAL TISSUE

To ascertain if MI/N1 reacts with either human foetal or adult brain, aliquots of an ascites preparation of the antibody were absorbed with equivalent amounts of these tissues. As a control for nonspecific absorption onto brain tissue, a similar incubation of antibody with rat brain was undertaken. The absorbed antisera were subsequently tested for residual binding activity with line CHP 100. The titration curves obtained (Fig. 4) show a single absorption with human foetal brain removed approximately 25% of the antibody activity whereas adult brain (cerebellum) absorbed 75% of the reactivity to CHP 100. MI/N1 did not react with adult rat brain because the titration curve obtained with this material was identical to that found for the unabsorbed antibody (Fig. 4).

MI/N1 reacted (by indirect immunofluorescence) with five of eight bone marrow biopsies heavily infiltrated with neuroblastoma cells; this result possibly reflects the heterogeneity seen in the binding of antibody to neuroblastoma cell lines. An ascites preparation from a child histologically shown to contain neuroblasts also reacted with the MI/N1, but the one retinoblastoma examined to date did not bind the antibody.

REACTIVITY WITH HAEMOPOIETIC CELLS

Inasmuch as one objective of producing a monoclonal antibody to neuroblastoma cells was to provide an immunodiagnostic aid for the detection of early metastatic spread in the bone marrow, it was clearly of some importance to examine critically the possible reactivity of MI/N1 with haemopoietic cells. That some reactivity to haemopoietic cells might be expected is indicated from the binding of the antibody to the cell lines, strongly to the myeloid line HL 60 and weakly to the T-cell leukaemia line HPB-TALL (Table 1). The monoclonal antibody, however, does not react with fresh human red blood cells.

A portion of the cells in normal bone marrow react by indirect immunofluorescence with MI/N1. When these were separated by FACS, it was evident that the cells were of the myeloid lineage at different stages of maturation. Lymphocytes and erythroid cells were found in the fluorescent negative fraction. Further separation by FACS of the fluorescent-positive granulocyte population showed that the brightest fraction of cells contained predominantly the most mature cells (neutrophils) (Table 2). Analysis of fractions of decreasingly fluorescent cell populations indicated that the earlier cells in the myeloid lineage expressed less antigen, with the promyelocytes and myelocytes in the dimmest fraction. Normoblastoid cells appeared in the fluorescent-negative population by FACS analysis (see Table 2).

The above observations are further confirmed by an analysis of leukaemic cells reacting with MI/N1. Although chronic myeloid leukaemias were shown to react with the antibody, the less mature cells found in acxute myeloid leukaemias were unreactive, as were the non-B, non-T common acute lymphoblastic leukaemias assayed. Of the five T-cell leukaemias examined, only one case was found to contain a population of cells binding MI/N1 (paralleling the binding to the line HPB-TALL).

DISCUSSION

The technique of somatic cell hybridisation has enabled the production of immunologic reagents that are monoclonal and directed against a single determinant; they have, therefore, a much more restricted specificity than most "conventional" polyspecific antisera (18, 19). Such reagents should be of considerable use for cell identification in complex or mixed populations, and to this end, an attempt has been made to produce a monoclonal reagent to neuroblastoma cells (12). A monoclonal antibody, MI/N1, is
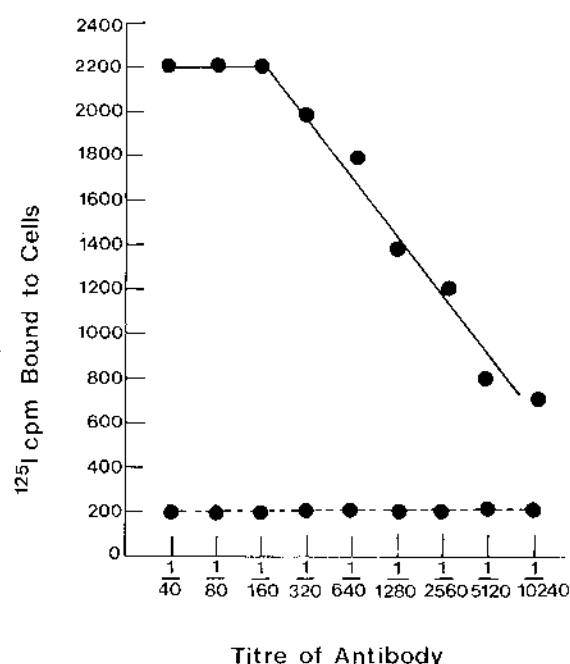
described that reacts with several neuroblastoma cell lines and five of eight bone marrow aspirates heavily infiltrated with neuroblastoma tumour cells.

Quantitative differences in the expression of antigen recognised by MI/N1 were observed between the neuroblastoma cell lines

Titre of Antibody

Fig. 3. The binding of MI/N1 to human and murine neuroblastoma cells. Two × 10^5 cells of each type were incubated with differing dilutions of an ascites preparation of MI/N1 followed by 100,000 cpm of ^{125}I-goat anti-mouse (see Materials and Methods). Iodinated antibody bound to the cells is plotted minus a background of 200 cpm obtained by incubating cells with the second antibody only. ●——●, binding to the human line CHP 100; ●– – –●, binding to the murine line ICRF N.1.
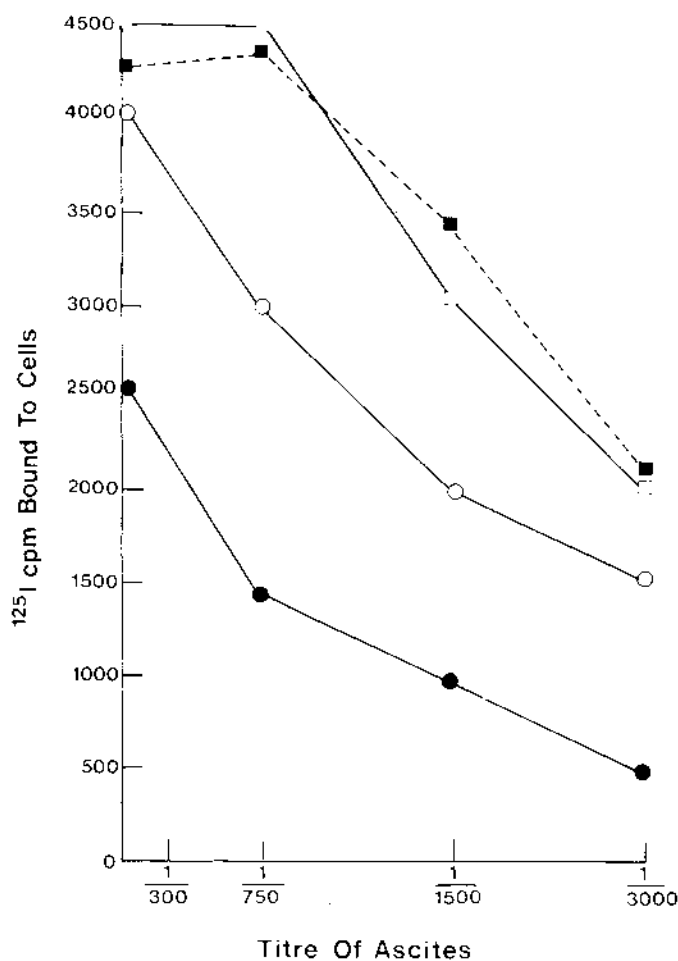

Titre Of Ascites

Fig. 4. The binding of MI/N1 to CHP 100 neuroblastoma cells after absorption with fresh neural tissue. MI/N1 diluted 1/100 was incubated with freshly prepared neural tissue as described in "Materials and Methods." Different dilutions of this material were subsequently incubated with 2 × 10^5 CHP 100 cells, followed by 100,000 cpm. ^{125}I-goat anti-mouse ("Materials and Methods") iodinated antibody bound to cells is plotted minus a background of 200 cpm obtained by incubating the cells with the second antibody only. – – – –, MI/N1 not absorbed; □, absorbed rat foetal brain; ○, absorbed human foetal brain (16 wk); ■, absorbed human adult cerebellum.

Table 2. *Analysis of cells binding MI/N1 in the bone marrow*[1]

		Histologic characterisation of cells			
Fraction	Fluorescent intensity	% myelocytes promyelocytes	% metamyelocytes	% neutrophils eosinophils	Other cell types
1	+++	3	47.5	49.5	
2	++	16	34	48.5	
3	+	43	24	29.5	3[2]
4	−ve	2		4	94[2]

[1] Red cells were lysed from the bone marrow sample by "flash lysis" using distilled water. The remaining cells (2 × 10^7) were incubated with MI/N1 and fluorescent F(ab')₂ goat anti-mouse F(ab')₂ as described in "Material and Methods." After separation by FACS of the populations of cells binding differing amounts of antibody, cytocentrifuge preparations of cells were obtained, and the slides were stained using May-Grunwald Giemsa.

[2] Pronormoblasts, basophilic normoblasts, polychromatic normoblasts, orthochromatic normoblasts, and lymphocytes in the preparation.

analysed (Fig. 1). One explanation for this observation is that cells in these lines have a maturation arrest at particular levels of neuronal development associated with distinctive antigenic changes (*cf.* haemopoietic malignancies) (13, 21). The expression of antigens or other markers on cell lines may not, however, be a true representation of that found on fresh tissue as the former represent highly selected populations growing only from stage 3 and 4 tumours (10) and may change their antigenic expression under prolonged periods in culture. MI/N1 shows, however, a similar heterogeneity in binding to fresh neuroblasts that have metastasised to the bone marrow. Of eight such marrow aspirates examined, only five appeared positive for MI/N1 binding by fluorescent microscopy. A variation in antigenic expression (both quantitative and qualitataive) may thus occur between different neuroblastoma tumours, again possibly reflecting a maturation arrest at different points during neuronal cell differentiation. Differential reactivity of fresh neuroblastoma samples to rabbit anti-neuroblastoma antiserum has been previously described (13). The antigen recognised by MI/N1 also appears to be increasingly expressed during foetal brain development (J. T. Kemshead, J. Fitschy, and M. Greaves, manuscript in preparation), further suggesting it may be a differentiation or maturation-linked antigen in neuronal cell development.

Heterogeneity of antigenic expression may exist within a single neuroblastoma cell line (*e.g.*, CHP 100), and this could not be directly linked to cell morphology or cell cycle status. Fractionation (by FACS) and subsequent culture of the fluorescent-positive and -negative cells show that these gradually revert to heterogenous populations of cells (J. T. Kemshead, unpublished observation).

MI/N1 does not react with a mouse neuroblastoma cell line or fresh neuronal tissue from either rats or mice, suggesting some species restriction in the expression of antigen. However, because the original immunisations of human neuroblastoma cells were undertaken in mice, nonreactivity to rodent neural tissue provides only equivocal evidence for restricted species expression. Other antibodies against neuroblastoma cells, raised in identical ways do not show such restriction (J. T. Kemshead, unpublished observation).

Biochemical analysis of the antigen is currently under investigation. Binding of antibody to neuroblastoma cells is not inhibited by either pure GM_1 or a mixture of glycosphingolipids, suggesting that the antigen is not a ganglioside. This contrasts with the monoclonal antibody described by Eisenbarth *et al.* (5) which reacts with a ganglioside on neural cell surfaces. Indirect evidence that MI/N1 does not react with highly conserved molecules such as glycolipids also comes from the restricted species reactivity found.

The reactivity of MI/N1 with relatively mature myeloid cells may appear to compromise its use in detecting malignant neuroblasts that have metastasised to the bone marrow. This problem may, however, be avoided because reactive myeloid cells are morphologically identifiable and can be counterstained with antimyeloid/granulocyte sera. A more serious limitation may be the heterogeneity of antigenic expression on neuroblastoma cells *in vivo*, a point currently under investigation. Another monoclonal antibody raised against neuroblastoma cells (PI 153/3) by Kennett and Gilbert (16) also cross-reacts with some haemopoietic cells. Unlike MI/N1, this antibody does not react with early cells of the myeloid lineage or with T cells but reacts with B cells and their progenitors and corresponding leukaemias (8). MI/N1 has no reactivity with B cells or their progenitors whereas its reactivity to T cells remains to be fully investigated.

Further attempts are being made to produce a monoclonal antibody with specificity restricted to normal and/or malignant

neural cells although in our hands almost all of the antibodies produced show some cross-reactivity with cells of the haemopoietic system.

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