Monoclonal antibody 3F8 recognises the neural cell adhesion molecule (NCAM) in addition to the ganglioside GD2

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Summary
The monoclonal antibody 3F8 has been described as binding to the ganglioside GD2. This antibody, of the IgG1 isotype, has been used in immunotherapy, radioimmunolocalisation and targeted radiation therapy. 3F8 was originally observed to have a binding profile similar to two monoclonal antibodies, UJ13A and 5.1.H11, characterised as binding to the neural cell adhesion molecule (NCAM). This observation has also been confirmed using a hetero-antiserum prepared against purified NCAM. The cross-reactivity of 3F8 with NCAM has been confirmed by cross-blocking studies with an anti-NCAM antiserum, and by direct immunoprecipitation and gel electrophoresis. In addition, we show that 3F8 binds to human NCAM from 3T3 fibroblasts transfected with NCAM cDNA constructs. It is possible that the common epitope shared by GD2, ganglioside and NCAM involves steric acid residues common to both the ganglioside and the glycoprotein.

Monoclonal antibodies have been widely employed in the search for tumour associated antigens. Many of these reagents have been shown to react with carbohydrate moieties associated with glycoproteins and/or glycolipids (Magnani, 1984; Feizi, 1985; Feizi & Childs, 1985). Gangliosides, in particular, appear to be particularly good markers for neuroectodermal tumours, GD2, for example, is present on human brain, but found in much higher amounts on melanoma and neuroblastoma tissues (Cahan et al., 1982; Schultz et al., 1984). Antibodies such as 3F8, recognised the GD2 ganglioside (Cheung et al., 1985; Saito et al., 1985), have been used for radioimmunoscinography studies (Cheung et al., 1986; Miraldi et al., 1986), and in phase 1 clinical trials (Cheung et al., 1987). Melanoma and neuroblastoma patients have been given 3F8, either alone or conjugated to ¹³¹I and some clinical responses have been noted.

We became particularly interested in the expression of GD2 as detected by monoclonal antibody 3F8, when it became apparent that the staining pattern of the antibody was similar to monoclonal antibodies UJ13A and 5.1.H11 (Kemshead, 1988) recently identified as binding to the neural cell adhesion molecule (NCAM) (Patel et al., 1989a, b). In fact, a variety of monoclonal antibodies to GD2 have been shown to interfere with neuroblastoma and melanoma cell attachment to various substrate adhesive proteins (Cheresh et al., 1986). Cheresh et al. (1986) suggest that GD2 and GD3 play a role in cell adhesion, excluding the possibility that the reagents studied are binding to glycoproteins.

We report here similarities in the staining profiles of 3F8 and an anti-NCAM antiserum and direct inhibition of 3F8 binding with the polyclonal antiserum. Monoclonal antibody 3F8 also immunoprecipitates a 145 kDa glycoprotein after labelling of cellular glycoproteins with ³⁵S methionine. In addition, the monoclonal antibody binds to 3T3 cells transfected with a human NCAM cDNA and Western blot analysis shows that the antibody reacts with the 125 kDa NCAM isoform in these cells.

Materials and methods

Cell lines
All cell lines, except those transfected with NCAM cDNAs, were grown at 37°C in a 6% CO₂ incubator using RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) (Gibco), 2mM glutamine (Gibco), 100 IU ml⁻¹ penicillin (Gibco) and 100 μg ml⁻¹ streptomycin (Gibco) (complete medium). Cells were harvested in their exponential growth phase for subsequent studies. 3T3 fibroblasts transfected with human NCAM cDNA were grown as described previously (Gower et al., 1988).

Antibodies and indirect immunofluorescence
Monoclonal antibody 3F8 was raised following immunisation of mice with neuroblastoma cells (Cheung et al., 1985). Two preparations of antibody were used for these studies. One was a purified fraction of ascites and the other concentrated tissue culture supernatant from the hydridoma. Monoclonal antibodies Thy-1 (Botmore et al., 1981), UJ13A (Allan et al., 1983) and 5.1.H11 (Hurko & Walsh, 1983), were supplied as purified antibodies from tissue culture supernatant (ICRF central antibody service laboratory). The hetero-antiserum to NCAM was raised by hyperimmunisation of rabbits with purified mouse muscle NCAM (Moore et al., 1987).

A pellet of 10⁶ cells or 6 μm cryostat sections were incubated with either sufficient anti-NCAM antiserum or antibody 3F8 to saturate antibody binding sites. After washing twice with phosphate buffered saline containing 1% FCS, the material was further incubated with an excess of either fluorescein conjugated goat anti-rabbit Ig or sheep anti-mouse Ig for 30 min. Cells/slides were subsequently washed twice and examined using a Zeiss fluorescence microscope with epi-illumination optics.

Cross-blocking studies
Some 2.5 x 10⁶ JR1 human rhabdomyosarcoma cells in 10 μl were plated into 96-well plates pre-blocked with protein. Fifty μl of 1:10 dilution of rabbit anti-mouse NCAM antiserum was added to the wells for 30 min. After washing twice, as indicated above, ¹²⁵I labelled 3F8 (10⁵ counts min⁻¹ well⁻¹) was added to the cells for a further 20 min. The plates were washed free of unbound 3F8 and material binding to the cell pellet estimated, using an LKB Ultra-gamma counter.

Metabolic labelling and antigen extraction
Medium from an exponentially growing flask of SK-N-SH neuroblastoma cells was replaced for 16 h with methionine free medium supplemented with ³⁵S methionine.
amounts the Ig containing phenylmethylsulphonylfluoride (PMSF) and 10 μg ml⁻¹ leupeptin, (lysis buffer) and lysed for 30 min at 4°C. Following centrifugation and pre-clearance with rabbit anti-mouse Ig bound to *Staphylococcus aureus* (Staph. a.) different amounts of either 3F8 or the hetero-antiserum were added to the extract (3 x 10⁵ cell equivalents) for 18 h. This was followed with a further 60 min incubation with either Staph. a. or Staph. a. pre-coated with rabbit anti-mouse Ig.

Immune complexes were pelleted by centrifugation and washed four times with lysis buffer. The final pellet was resuspended in 50 μl of SDS-sample buffer (10 mM Tris/HCl, pH 6.8, 10% glycerol and 2% SDS) and boiled for 3 min. On some occasions, cells were incubated with antibody before disruption in the lysis buffer. This was to allow cross-linking of the antibody to the membrane antigen. Cross-linking was achieved by incubating antibody coated cells in 2.0 ml of PBS (pH 8.3) containing 1 mM MgCl₂, 0.02% NaN₃ and 10 μM dithiobis (sucinimidy l propionate). After one hour at room temperature, the cells were centrifuged and resuspended in 100 mM Tris buffer pH 8.0 containing 140 mM NaCl. Following centrifugation, the cells were lysed in NP40 containing buffer and the extracts treated as described above.

**SDS-page**

Slab polyacrylamide gel electrophoresis was carried out according to the method of Laemmli (1970). The gels were stained with Coommasie Blue and unprogressed with ‘Amplify’. Autoradiography was carried out at ~70°C using Fuji RX X-ray film and intensifying screens. Autoradiograms were developed 1–3 days after exposure to the gel.

**Immunoprecipitation and Western blotting**

Immunoprecipitation and Western blot analysis of 3T3 fibroblasts transfected with a cDNA coding for 125 kDa human muscle NCAM isofrom (G4 cells) was carried out with monoclonal antibody 3F8 as described previously (Gower et al., 1988).

**Results**

**Binding of an anti-mouse NCAM antiserum and monoclonal antibody 3F8**

We have compared the staining profiles of a rabbit anti-mouse NCAM antiserum and monoclonal antibody 3F8 on a variety of tissues and cell lines. Similar binding patterns were identified on a variety of neuroectodermal and mesodermal tissues. Neuroblastomas, Wilms’ tumours, rhabdomyosarcomas and Ewing’s tumours stained equally well with the anti-NCAM antiserum and the monoclonal antibody 3F8 (Table I). Fetal brain and fetal muscle also bound both reagents. Adult brain reacted with the hetero-antiserum and monoclonal antibody 3F8 but no binding to adult skeletal, smooth or cardiac muscle was observed. In addition, fetal liver, adult spleen, thymus and retina did not bind either 3F8 or the anti-NCAM antiserum.

A high degree of concordance was also observed on screening a variety of cell lines with both reagents. Both monoclonal antibody 3F8 and the anti-NCAM antiserum bound 6/8 human neuroblastoma lines tested (Table II). The PCF and SK-N-LO lines did not bind either antibody, although by other criteria these are clearly neuroblastoma cell lines. In addition, the human rhabdomyosarcoma cell line JR1 bound both monoclonal antibody 3F8 and the anti-NCAM antiserum. In the majority of these cell lines more than 90% of cells bound the two reagents, although there were differences in the intensity of staining noted. Less than 20% of the neuroblastoma lines GOTO and NB1 bound antibody 3F8. This compared to 90% of the cells binding the anti-NCAM antiserum, which may again reflect quantitative differences in the binding of the two antibody preparations. In all cases binding was restricted to these primitive embryonic tumour cell lines as a variety of haemopoietic lines did not bind either reagent (Table II). Similar results were obtained using 3F8 obtained from either ascites or culture supernatant. In addition, this data agrees with the specificity of antibody 3F8 obtained in the neuroblastoma workshop using a coded preparation of culture supernatant.

**Anti-NCAM antiserum inhibits the binding of monoclonal antibody 3F8 to the JR1 rhabdomyosarcoma cell line**

To substantiate further that the monoclonal antibody 3F8 could bind to NCAM, blocking studies were undertaken. Pre-incubation of JR1 cells with anti-NCAM antiserum (see Figure 4) reduced the level of 3F8 binding (prepared from either ascites or tissue culture fluid) to approximately 10% of that seen in the control (Figure 1). As might be expected preincubation of cells with an excess of monoclonal antibody 3F8 (ascites preparation) also inhibited the binding of radiolabelled monoclonal antibody to the cell line. In contrast to these results, anti-NCAM antiserum did not block the binding of two other monoclonal antibodies (U15A4 and 5.1.H11) to JR1 cells (Figure 1). Furthermore the antiserum did not block the binding of an antibody to an irrelevant

**Table I Reactivity of anti-NCAM antibody and monoclonal antibody 3F8 to tissues**

| Tissues          | Anti-NCAM | 3F8 |
|------------------|-----------|-----|
| Tumours          |           |     |
| Neuroblastoma    | 4/4       | 4/4 |
| Wilms’ tumour    | 2/2       | 2/2 |
| Medulloblastoma   | 1/1       | 1/1 |
| Ewing’s tumour   | 4/4       | 4/4 |
| Rhabdomyosarcomas| 6/6       | 6/6 |
| Fetal tissues    |           |     |
| Brain            | 1/1       | 1/1 |
| Skeletal muscle  | 1/1       | 1/1 |
| Heart muscle     | 0/1       | 0/1 |
| Liver            | 0/1       | 0/1 |
| Adult tissues    |           |     |
| Brain            | 3/3       | 2/2 |
| Skeletal muscle  | 0/2       | 0/2 |
| Smooth muscle    | 0/2       | 0/2 |
| Cardiac muscle   | 0/2       | 0/2 |
| Liver            | 0/4       | 0/4 |
| Thymus           | 0/1       | 0/1 |

*The number of samples positive per number tested.

**Table II Reactivity of anti-NCAM antibody and monoclonal antibody 3F8 against a variety of human cell lines**

| Cell line          | Anti-NCAM | 3F8 |
|--------------------|-----------|-----|
| Neuroblastoma      |           |     |
| Kelly              | 2+        | 3+  |
| IMR-32             | 1+        | 2+  |
| SK-N-SH            | 2+        | 3+  |
| PCF                |           |     |
| GOTO               | 1+        | 1+  |
| SK-N-LO            | 2+        | 1+  |
| NB1                | 2+        | 1+  |
| CHP 100            | 1+        | 1+  |
| Rhabdomyosarcoma   |           |     |
| JR1                | 2+        | 3+  |
| Haemopoietic       |           |     |
| HL60               |           |     |
| K562               |           |     |
| CCRF-CEM           |           |     |

*Arbitrary binding units: 3+, strongly positive (>90% Cells binding antibody); 2+, weakly positive (75-90% Cells binding antibody); 1+, weakly positive (50-75% Cells binding antibody); 0, negative. *Less than 20% of cells positive. *Schwab et al., 1983; *Tumilowicz et al., 1970; *Biedler et al., 1973; *Kemenshead et al., 1988; *Sekiiguchi et al., 1979; *Sugimoto et al., 1984; *Imashuku et al., 1973; *Schlesinger et al., 1976; *Clayton et al., 1986; *Collins et al., 1977; *Anderson et al., 1979; *Morikawa et al., 1978.
antigen (Thy-1) present on the JR1 cell line. In a further series of blocking experiments we have confirmed that the epitopes present on NCAM that are recognised by UJ13A, 5.1.H11, ERIC-1 and 3F8 are distinct (Patel et al., 1989a).

**Biochemical characterisation of the binding of monoclonal antibody 3F8 to either neuroblastoma or rhabdomyosarcoma cell lines**

As has been previously reported, monoclonal antibody 3F8 binds to the ganglioside GD₃ (Cheung et al., 1985). This we have confirmed following chloroform:methanol:water extraction of glycolipids from rhabdomyosarcoma cells, thin layer chromatography, immunoblotting with ¹²⁵I labelled 3F8 antibody and autoradiography (data not presented).

To determine if the epitope recognised by 3F8 involves sialic acid sugar moieties, JR1 cells were treated with neuraminidase. This reduced the level of radiolabelled antibody 3F8 (from culture supernatant) binding to 20% of the control as determined in a radiobinding assay. In contrast, no diminution of monoclonal antibody 5.1.H11 binding to JR1 cells was observed after a similar treatment of cells with neuraminidase (Figure 2).

Surface labelling of either the SK-N-SH or JR1 cells with ¹²⁵I using the lactoperoxidase technique (Marchalonis et al., 1971), cell lysis in extraction buffer (0.1 M Tris/HCl pH 7.4 containing 0.9% NaCl, 0.5% Nonidet P-40, 2 mM phenyl methyl sulphonylfluoride), centrifugation (x 100,000 g for 30 min) and immunoprecipitation (Kemshhead et al., 1983) did not result in the identification of any glycoprotein binding to monoclonal antibody 3F8. A similar result was found after Western blotting cell extracts (either NP40 or SDS extracts) of either neuroblastoma or rhabdomyosarcoma cells. However, after metabolically labelling neuroblastoma cells with ³⁵S methionine and immunoprecipitation with 3F8, two major bands could be identified at 145 and 65 kDa along with an occasional weak band at 120 kDa (Figure 3). The 145 and 120 kDa bands correspond to two of the isoforms of NCAM often reported after immunoprecipitation with the relevant antiserum. Similar bands of 145 and 65 kDa were identified following immunoprecipitation of ³⁵S labelled JR1 extracts (data not shown). In addition, a similar band of 145 kDa was identified following cross-linking and immunoprecipitation of ³⁵S labelled cells, with an antiserum to mouse NCAM (Figure 3).

In contrast to this, no bands at these molecular weights were identified using either a control monoclonal antibody (anti-Thy) known to bind to the human homologue of the Thy-1 antigen (Cotmore et al., 1981) or a non-immune rabbit antiserum.

**Reactivity of 3F8 and 3T3 fibroblasts transfected with human NCAM cDNA**

cDNA clones corresponding to the full coding sequence of various NCAM isoforms from human muscle have recently been isolated and placed in appropriate expression vectors (Gower et al., 1988). Subsequently, the cDNAs have been

![Figure 1](image1.png)

**Figure 1** Anti-NCAM antiserum blocks the binding of 3F8 to target cells. 2 x 10⁵ JR1 rhabdomyosarcoma cells were incubated for 30 min with either a hetero-antiserum to mouse NCAM or a non-immune serum (similar protein concentrations). After washing, cells were further incubated with either 100,000 c.p.m. of ¹²⁵I labelled monoclonal antibody 3F8 or a series of other radiolabelled monoclonal antibodies. Monoclonal antibody binding to cells was assessed after washing using an LKB Ultragamma counter. Results illustrate one experiment of three. Mean values of triplicate assay points presented. a, Cells pre-incubated with non-immune serum and ³⁵S antibody 3F8 (100% binding); b, Cells pre-incubated with 3F8 antiserum and ³⁵S antibody 3F8; c, Cells pre-incubated with an excess of anti-NCAM antiserum and ³⁵S antibody 3F8; d, Cells pre-incubated with non-immune serum and ³⁵S antibody 5.1.H11 (anti-NCAM) (100% binding). e, Cell pre-incubated with 3F8 monoclonal antibody and ³⁵S antibody 5.1.H11 (anti-NCAM). f, Cells pre-incubated with non-immune serum and ³⁵I antibody UJ13A (anti-NCAM) (100% binding). g, Cells pre-incubated with 3F8 antiserum and ³⁵I antibody UJ13A (anti-NCAM). h, Cells pre-incubated with non-immune serum and ³⁵I antibody Thy-1 (anti-Thy-I) (100% binding). i, Cells pre-incubated with 3F8 antiserum and ³⁵I antibody Thy-1 (anti-Thy-I).

![Figure 2](image2.png)

**Figure 2** 3F8 binding is inhibited by neuraminidase treatment of target cells. 5 x 10⁵ JR1 cells in 1 ml of 50 mM acetate buffer pH 5.0 containing 150 mM NaCl were exposed to 1 IU ml⁻¹ of neuraminidase (from Clostridium Perfringens) for 1 h at 37°C. After washing x 2 with PBS containing 1% bovine serum albumin, cells were incubated with an excess of monoclonal antibody 3F8 for 30 min at room temperature. Excess antibody was removed by washing x 2 and cells were incubated with 100,000 c.p.m. ¹²⁵I sheep anti-mouse Ig. Antibody binding to the cells was determined after washing using an LKB Ultra-gamma counter. Results illustrate one experiment of three. Mean values of triplicate assay points presented. □, − neuraminidase; △, + neuraminidase.
transfected into 3T3 fibroblasts and permanent cell lines obtained. One of these (called G4) which synthesises a 125 kDa NCAM isofrom (Gower et al., 1988) was analysed for 3F8 antibody binding. Control experiments showed that 3T3 fibroblasts did not express detectable levels of NCAM (Gower et al., 1988). NCAM could be immunoprecipitated from the G4 transfectants using an anti-human NCAM antiserum. When this material was subsequently Western blotted protein bands of 110 and 125 kDa could be identified following incubation of the blots with the anti-NCAM antiserum (Figure 4a). Incubating similar blots with the monoclonal antibody 3F8 also resolves a band of 125 kDa with weaker binding to the 110 kDa protein. Incubation of the Blots with either an irrelevant monoclonal antibody or non-immune antiserum gave no signal in the 100–150 kDa range.

Discussion

Cross-reactions of monoclonal antibodies with identical or similar epitopes on different structures have been reported previously. Usually carbohydrate epitopes are involved. Whilst these cross-reactions mainly involve the monoclonal antibody they occasionally can be due to naturally occurring anti-carbohydrate antibodies present to differing degrees in different ascites preparations. To exclude the possibility that the cross-reaction of the antibody 3F8 with NCAM is due to these anti-carbohydrate specificities many of the experiments reported in the text have been undertaken with antibody prepared from both ascites and tissue culture fluid.

The epitope on GD3 recognised by monoclonal antibody 3F8 involves sialic acid residues as demonstrated by the inhibition of binding as a result of treatment of target cells with neuraminidase. Sialic acids are linked by alpha 2–3 and alpha 2–8 linkages in GD2. The latter linkage is generally not found in mammalian glycoproteins. However, it does occur in NCAM, a glycoprotein that is both sialylated and polysialylated on the extracellular portion of the molecule to varying degrees (Cunningham et al., 1983; Finne et al., 1983).

Cheresh et al. (1986) have proposed a role for GD3 in cell adhesion since antibodies to GD3 interfere with cell attachment. In their study, they excluded the possibility of monoclonal antibodies 3F8 and 126.4 (both anti-GD3) binding to glycoproteins rather than GD3 on the basis that the antibodies did not immunoprecipitate a protein from metabolically labelled cells nor did they react by Western blot analysis with any protein. While we would agree that monoclonal antibody 3F8 does not immunoprecipitate radiolabelled glycoproteins or identify proteins by Western blot analysis, our data show that the reagent recognises a 145 kDa glycoprotein following metabolic labelling of cellular glycoproteins with 35S methionine. The inhibition of cell adhesion seen with a variety of anti-GD3 antibodies, including monoclonal antibody 3F8 could, therefore, be due to a cross-reactivity with NCAM.

Immunoprecipitation of 35S methionine labelled cell extracts with monoclonal antibody 3F8 revealed two major bands of 145 and 65 kDa and a minor band of 120 kDa. The 65 kDa band is possibly a degradation product of NCAM (Hoffman et al., 1982). The 145 kDa and 120 kDa bands correspond to two of the three isofoms of NCAM (reviewed by Cunningham et al., 1987). We exclude the possibility that GD3 exists in a complex with NCAM since it is likely that the extraction conditions used would have disrupted lipid–protein interactions. Further confirmation that monoclonal antibody 3F8 cross-reacts with NCAM was obtained from immunoprecipitation and Western blot analysis of fibroblasts transfected with human muscle NCAM. Monoclonal antibody 3F8 binds to immunoprecipitated NCAM from 3T3 cells transfected with a human NCAM cDNA. While the post-translational modification of human NCAM in 3T3 cells cannot fully mimic that which occurs in the muscle cell, sufficient glycosylation takes place to allow binding of the 3F8 antibody and other anti-NCAM antibodies.
that are thought to bind to carbohydrate moieties (Patel et al., 1989a). The glycosylation pattern of the transfected protein is not fully understood although the lack of binding of a monoclonal antibody to alpha-2 linked polylactic acid chains (greater than eight residues) would suggest that this modification does not take place in 3T3 cells.

The fact that all three isoforms of NCAM are not detected by monoclonal antibody 3F8 in JR1 cell extracts might reflect differential glycosylation of the three isoforms. Alternatively, it is known that the 145 kDa form of NCAM is the earliest isoform expressed during embryogenesis (Levi et al., 1987) and it is therefore, not surprising that the 145 kDa isoform is predominantly detected in a primitive embryonic tumour such as rhabdomyosarcoma. Northern blot analysis of RNA extract from the SK-N-SH neuroblastoma and JR1 rhabdomyosarcoma cell line would suggest that this is the case as a 6.7 kb mRNA representing the 145 kDa protein (Dickson et al., 1987; Santoni et al., 1987; Small et al., 1987) is detected with a human NCAM cDNA probe. In contrast, cell lines which were negative for monoclonal antibody 3F8 and anti-NCAM antiserum staining did not contain NCAM mRNA as detected by Northern blot analysis (data not presented).

Other monoclonal antibodies which cross-react with NCAM have been described. One of the best characterised is the HNK-1 antibody (Albo & Balch, 1981). This antibody appears to recognise sulphated carbohydrate (Chou et al., 1985, 1987; Shashoua et al., 1986) present on a number of molecules involved in adhesion, including NCAM (Kruse et al., 1984, 1985; McGarry et al., 1983). The HNK-1 determinant is present on only 15–20% of all NCAM molecules (Kruse et al., 1984). The epitopes recognised by monoclonal antibodies 3F8 and HNK-1 are different since, in an international workshop on neuroblastoma, there was considerable difference in the staining profiles of the two antibodies (Kemshead, 1988). Furthermore, the HNK-1 epitope differs from that recognised by 3F8 in that it has been reported as not involving neuraminic acid (Chou et al., 1985).

In conclusion, we have evidence that monoclonal antibody 3F8 reacts with NCAM in addition to its reactivity against GD2. Since monoclonal antibody 3F8 has been used for clinical trials on the basis that it was reactive against only GD2, its binding specificity needs to be re-examined. Cross-reactants should now needs to be re-determined in view of such monoclonal antibody 3FS for clinical purposes, but it will be necessary to evaluate them fully so that accurate binding profiles, affinity constants and antigen densities can be compared and contrasted.

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