Topography of N-CAM Structural and Functional Determinants. I. Classification of Monoclonal Antibody Epitopes

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Abstract. 12 distinct neural cell adhesion molecule (N-CAM) epitopes, each recognized by a different monoclonal antibody (mAb), have been characterized in terms of the major structural and functional features of the molecule. Seven antibodies, each recognizing the amino-terminal region of the molecule, altered the rate of N-CAM–mediated adhesion. Four of these were inhibitors, two of which also recognized a heparin-binding N-CAM fragment. The other three antibodies specifically enhanced the rate of N-CAM–mediated adhesion. Three epitopes, one polypeptide- and two carbohydrate-dependent, were associated with the sialic acid–rich central portion of the molecule. The remaining two antibodies were found to react with intracellular determinants, and are specific for the largest of the three major N-CAM polypeptide forms. Studies on the ability of one antibody to hinder recognition of native N-CAM by another antibody suggested that the epitopes associated with N-CAM binding functions are in close proximity compared with the other determinants. The classification of these mAb epitopes has allowed the topographical placement of key N-CAM features, as described in the following paper, and provides valuable probes for analysis of both the structure and function of N-CAM.

NEURAL cell adhesion molecule (N-CAM)[1] is a membrane glycoprotein that serves as a homophilic ligand in the formation of adhesions between cells (29). This molecule is expressed by several types of cells during embryogenesis, including nerve, muscle, and glial cells of the developing nervous system (see reference 26). N-CAM–mediated adhesion is involved in a variety of developmental events, including axon guidance (32, 34), segregation of cells into discrete regions and layers (1, 30), and the formation and innervation of muscles (28, 35). The ability of the molecule to contribute to the specificity of these intricate structures and processes is reflected not only in an elaborate spatial and temporal regulation of its expression, but also in variations in its polypeptide and carbohydrate structure (26, 27).

Significant progress has been made in describing some of the major chemical properties of N-CAM, as well as the genetic basis for generating its different polypeptide forms (27). In integrating this information into the biology of cell-cell adhesion, it is useful to create models that relate these properties to each other and to the overall function of N-CAM, and to obtain specific probes for individual molecular structures and functions. For a number of large polypeptides, an effective approach to both of these goals has been to generate a library of monoclonal antibodies (mAbs) selected and characterized with respect to their ability to recognize or perturb specific domains and activities (9, 11, 13, 19, 22). In this study we have established such a library for N-CAM. In the accompanying report (10) the associated epitopes are mapped into a molecular model of this transmembrane glycoprotein.

1. *Abbreviations used in this paper*: Endo-F,-N, endoneuraminidase F and N; N-CAM, neural cell adhesion molecule.

Materials and Methods

Electrophoresis and Immunoblotting

Samples were boiled for 3 min in SDS sample buffer and separated by electrophoresis in 7, 7.5, 10, or 15% polyacrylamide gels containing SDS (20). Gels were silver stained according to the method of Oakley et al. (24) or transferred to nitrocellulose for immunoblotting (36). For the blots, ascites fluid or monoclonal IgG were diluted in Blotto (18) and incubated with the sample for at least 3 h at room temperature. Unreacted antibody was removed by a series of four 5-min washes in a phosphate buffer (36). Bound antibody was reacted with ^{125}I-affinity-purified second antibody (New England Nuclear, Boston, MA or Amersham Corp., Arlington Heights, IL) and visualized by autoradiography on Kodak X-Omat AR x-ray film with an enhancing screen (33) or with peroxidase-conjugated goat anti-mouse Ig and visualized by development with 4-chloro-1-napthol (16).

Preparation of N-CAM

For preparation of N-CAM fragments and for production of mAbs N-CAM was purified by immunoaffinity chromatography as previously described (17), using monoclonal PP coupled to Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ). For purposes of immunoblotting with different mAbs, the three major molecular mass forms of N-CAM present in sonication-disrupted 2.5-wk-old chicken optic nerve, chiasm, and tract tissue (1 vol tissue/5 vol PBS) were partially desialylated by treatment of the sonicate with endoneuraminidase N (31) (endo-N; 1 μl/3 μg tissue for 1 h at 4°C) and separated by SDS PAGE. The endo-N digestion was stopped by addition of an equal volume of 2× SDS PAGE sample buffer and boiling for 3 min.

Preparation of Anti-N-CAM mAbs

mAbs 10A, 11B, 9A, 12B, 79B, 5E, 30B, and 4D were produced against purified N-CAM using published procedures (25) with the following modifications. BALB/C mice (Charles River Breeding Laboratories, Inc., Wilmington, MA) were immunized with 20–30 μg of N-CAM in complete Freund's adjuvant. The mice were boosted three to five times with 10–20 μg N-CAM in incomplete Freund's adjuvant at 1-mo intervals. 3–4 d before fusion, immune mice were given an intraperitoneal injection of 50 μg of N-CAM in PBS. Spleen cells were harvested, fused with a nonsecreting myeloma cell line (P3X63-Ag8.653) and hybrids selected by published procedures (25).

Media from hybrid clone cultures were tested for anti-N-CAM antibody by the solid-phase ELISA (37) using undenatured, purified N-CAM and o-phenylenediamine as substrate for the peroxidase. Cultures giving a positive reaction against undenatured N-CAM were cloned by limiting dilution (25) to ensure that they were monoclonal. Ascites fluid was produced for a number of hybridomas by injecting 5–10 × 10^6 cells into pristane-primed BALB/C mice and collecting fluid 10–30 d later (2).

Antibodies PP and CHO (34), 104 (gift of Drs. Sigrid Henke-Fahle and Friedrich Bonhoeffer, Institute for Developmental Biology, Federal Republic of Germany) and 105 (21; gift of Dr. David Gottlieb, Washington University School of Medicine, St. Louis, MO) were obtained by similar procedures, except that neural membranes or cells were used as the immunogen. The ability of these reagents to recognize epitopes on N-CAM was established either by the immunoblot pattern obtained with SDS PAGE of neural tissue or by their binding to purified N-CAM in the ELISA assay.

The immunoglobulin subclass of each mAb was determined with a kit from Boehringer Mannheim Biochemicals (Indianapolis, IN) according to manufacturers directions. The mAbs used for the following studies were found to be gamma-1/kappa, except CHO, which was mu/kappa (data not shown).

The IgG fraction of the ascites fluids was prepared either by dialysis against 20 mM Tris, pH 7.2, followed by chromatography on Affi-Gel Blue (Bio-Rad Laboratories, Richmond, CA) or by ammonium sulfate precipitation, dialysis, and chromatography on DE-52 (Whatman, Inc., Clifton, NJ). Affi-Gel Blue columns were eluted sequentially with the same buffer containing 100 mM NaCl. DE-52 columns were eluted with a continuous gradient of NaCl from 0 to 50 mM in the starting buffer, 25 mM sodium phosphate, pH 7.5. Purity of Ig was monitored by SDS PAGE using 15% gels and silver stain. Fab fragments of each mAb were prepared from the purified Ig by digestion with papain (200:1 wt/wt of Ig to papain) for 6–12 h at 37°C (23) so that no intact heavy chain was detectable.

Membrane Vesicle Aggregation

The ability of Fab fragments of the isolated mAbs to affect aggregation of brain membrane vesicles prepared from E14 chicken brain was analyzed by determining the rate of decrease in membrane particle number using a particle counter (ZB1; Coulter Electronics, Inc., Hialeah, FL) with a 100-μm aperture tube (31). Membrane vesicles were prepared on the day of the assay as previously described (17). Vesicles, 15–20 μl of a 10% vol/vol suspension in PBS, were incubated in a total volume of 50–100 μl with 25–100 μg of either control nonimmune Fab or 25–100 μg immune Fab for 15 min at 0°C. To initiate aggregation, sample volume was increased to 0.5 ml with 37°C PBS. Aggregation was carried out at 37°C at 70 rpm rotary oscillation in 20-ml glass scintillation vials, and stopped at timed intervals by dilution of 50 μl reaction mixture into 15 ml 1% glutaraldehyde in PBS.

Immunostaining of Intact and Permeabilized Cells

Retinal cells were prepared from neural retina of 9–11-d chick embryos by trypsinization (0.25% trypsin for 15 min in 0.02% EDTA in calcium and magnesium-free medium) followed by washing with PBS containing soybean trypsin inhibitor (0.052% inhibitor, 0.3% BSA, 0.004% DNAse) and dispersal of the cells with a pipette.

Staining was carried out both with the freshly trypsinized cells and with cells that had been cultured. The freshly trypsinized cells were kept at 4°C while being immobilized by centrifugation onto glass cover slips coated with waxbean agglutinin (15) and then immediately stained with antibody. Thus, the cells were never allowed to replace surface components cleaved by the original trypsinization. The cultured cells were prepared by plating on acid-washed, polylysine-coated glass coverslips (5 × 10^5 cells/coverslip) and culturing for 2 d in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) with 10% FCS.

Staining was carried out both with and without permeabilization of the cells by treatment with methanol at −20°C for 5 min. The cells were incubated for 30 min at 4°C with the indicated mAb diluted in PBS containing 10% normal goat serum. The cells were then washed five times with Hanks' balanced salt solution, incubated 30 min at room temperature in fluorescein-conjugated goat-anti mouse IgG (Cooper Biomedical, Inc., Malvern, PA), washed again, and fixed in −20°C methanol for 5 min. The coverslips were mounted on slides using 2% *n*-propyl gallate in glycerol to reduce photobleaching.

Binding of mAbs to Membrane Vesicles

The effect of preincubation of brain membrane vesicles with one mAb on the subsequent binding of a second ^{125}I-labeled antibody was used as a criterion for epitope proximity on native, membrane-associated N-CAM. Purified Ig or concentrated ascites fluid were iodinated by a modification of the Enzymo-Bead method (Bio-Rad Laboratories). 50 μl of a 2 mg/ml Ig solution or 50 μl of ascites fluid was incubated with 10 μl (0.5 mCi) ^{125}I, 50 μl rehydrated Enzymo-Beads, and 25 μl 1% D-glucose for 30 min. The iodination was stopped by dilution with 1 ml PBS. Ion exchange resin (AG1-X8, chloride form; Bio-Rad Laboratories) was added to adsorb unreacted iodine.

Binding and competition experiments were performed with freshly prepared 14-d chick embryo brain membrane vesicles (17). Vesicles (100 μl of a 1% wt/vol solution) were incubated in 12 × 75-mm test tubes for 15 min on ice with nonradioactive antibody (50 μg in 50 μl PBS containing 10% horse serum and 3% BSA) and an additional 15 min with iodinated antibody (50,000 cpm/50 μl). Samples were then diluted with 1 ml PBS, pelleted at 2,800 rpm for 15 min at 4°C, and assayed for bound radioactivity.

Preparation of the Amino-terminal 65-kD Fragment

The 65-kD Fr1 fragment of N-CAM was prepared as previously described (17). Briefly, this involves incubation of twice immunoaffinity-purified N-CAM at 37°C for 96–115 h in sodium bicarbonate buffer, pH 8.3, containing 0.1% sodium azide, followed by gel filtration on Sephacryl S-300 in the same buffer. The included peak was pooled, lyophilized, rechromatographed on the same column, and then immunoaffinity-purified (17) using monoclonal PP coupled to Sepharose 4B.

Preparation of a 25-kD Heparin-binding Fragment

A 25–29-kD subtilisin fragment of N-CAM, isolated by affinity chromatography on heparin-agarose (Sigma Chemical Co., St. Louis, MO), was generously provided by Drs. Greg Cole and Luis Glaser, Washington University, St. Louis, MO. This fragment can be used as a substrate for attachment of cells to plastic surfaces and also perturbs N-CAM-mediated cell-cell adhesion (3, 4).

Preparation and Characterization of a Polysialic Acid-containing Fragment

A cyanogen bromide (CNBr) fragment of N-CAM that carries the majority of the molecule's sialic acid was prepared by published procedures (5). Affinity-purified N-CAM was reduced and alkylated, then subjected to CNBr cleavage (8 mg N-CAM, 450 mg CNBr in 9 ml 70% formic acid) for 45 min at room temperature. The sample was diluted with 100 ml H_2O, lyophilized to remove unreacted CNBr, and the digest fractionated by gel filtration on a Sephacryl S-300 column equilibrated with 0.1 M ammonium bicarbonate. Column fractions were analyzed for sialic acid by the method of Warren (38) as modified by Hahn et al. (14). Fractions containing the included peak of sialic acid were pooled, concentrated by lyophilization, and treated with *Vibrio cholera* neuraminidase (Calbiochem-Behring Corp., La Jolla, CA), endo-F (gift of Drs. John Elder and Steve Alexander, Scripps Clinic and Research Foundation, San Diego, CA), or endo-N (gift of Dr. Eric Vimr, University of Illinois at Urbana-Champaign). The samples were then separated by SDS PAGE and the gels either silver stained or blotted and stained with mAbs. *Vibrio cholera* neuraminidase digestions were carried out at pH 5.8 in 0.1 M sodium acetate, 0.2 mM EDTA, and 2 mM calcium chloride for 16 h at 37°C (1 U neuraminidase/300 μg sialic acid). Endo-N digests were carried out for 60 min at 4°C in PBS containing 1.5 U enzyme/3 μg sialic acid. Endo-F digestions (6 U enzyme/3 μg sialic acid) were performed on material that had been boiled 2 min after a 1:1 dilution in pH 6.1 buffer containing 100 mM sodium phosphate, 50 mM EDTA, 1% NP-40, 1% mercaptoethanol, and 0.1% SDS (7).

Results

From a collection of over 100 hybridoma clones whose supernatants reacted with undenatured N-CAM, 12 antibodies were selected for their ability to recognize, in SDS PAGE immunoblots, epitopes associated with both intact N-CAM and proteolytic fragments of the molecule. These reagents have been assigned the trivial names 79B, 5E, 12B, PP, 9A, 11B, 10A, 104, 105, CHO, 30B, and 4D. The selected hybridomas were subcloned by limiting dilution, and again shown to produce antibodies reactive with purified N-CAM. These clones were injected into mice to produce ascites fluid, each of which only contained N-CAM–reactive antibodies of a single subtype. Together, these procedures and analyses were used as criteria that the antibodies were in fact monoclonal with respect to N-CAM antigenic determinants. The antibodies were then tested for several properties: (*a*) the ability to alter the rate of brain membrane vesicle aggregation, (*b*) reaction with extra- and intracellular epitopes, (*c*) inhibition among the antibodies for binding to native N-CAM, (*d*) specificity for carbohydrate versus protein determinants, and (*e*) recognition of fragments representing several defined N-CAM structural and functional domains.

Effects of the mAbs on Membrane–Membrane Adhesion

To avoid distortion of this assay by IgG-mediated agglutination of the membrane vesicles, each antibody tested was cleaved into the monovalent Fab form and analyzed by electrophoresis. If heavy chain was still detectable, the cleavage was repeated until complete (Fig. 1). The effect of these Fab fragments on the rate of brain membrane vesicle aggregation, relative to the rate observed in the presence of nonimmune Fab, is shown in Fig. 2. Based on these results the antibodies could be divided into three groups: those with no effect (4D, 30B, and 105), those that decreased the rate of aggregation (79B, 5E, 12B, and PP; t test $P < 0.005$), and those that enhanced aggregation (9A, 11B; t test $P < 0.005$).

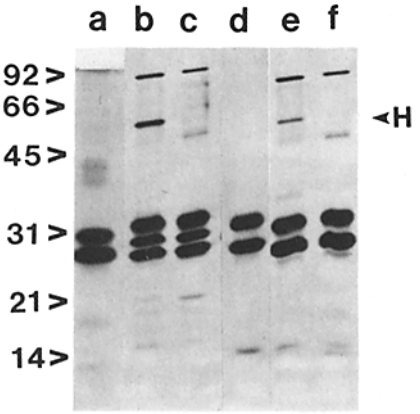

Figure 1. Representative SDS PAGE analyses of Fab fragments used in the adhesion assay shown in Fig. 2. The silver-stained components of representative monoclonal IgGs after papain digestion are shown: lane *a*, 5E; lane *b*, 11B after one digestion; lane *c*, 11B 6 h after two digestions; lane *d*, 105; lane *e*, 30B after one digestion; and lane *f*, 30B after two digestions. When heavy chain was still detected after one digestion, as with 11B (lane *b*) and 30B (lane *e*), the cleavage was repeated (lanes *c* and *f*). Comparable results were obtained with the other antibodies used in the adhesion assay.

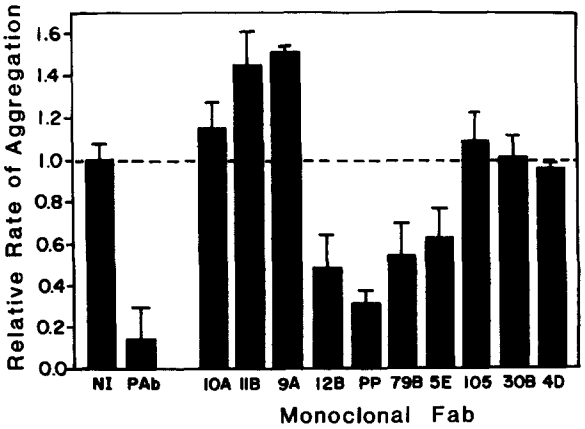

Figure 2. The effect of anti–N-CAM Fabs on the rate of brain membrane vesicle aggregation. Fab fragments of polyclonal and monoclonal antibodies were added to the vesicles and the initial rate of aggregation at 37°C was monitored with a particle counter. 100% aggregation was taken as the percent decrease in particle number (typically 40%) after 20 min in the presence of the control nonimmune (NI) Fab. Each Fab was tested 4–12 times and the standard error of the mean of these determinations is shown. The monoclonal Fabs could be placed into three categories: those that inhibited (12B, PP, 79B, and 5E), those that enhanced (11B, 9A, and 10A), and those that had no effect (105, 30B, and 4D) on aggregation. Combinations of the inhibitory monoclonal Fabs blocked aggregation up to 80% (12B plus 5E), but never as well as rabbit polyclonal anti–N-CAM Fab (PAb).

Antibody 10A caused a weaker but significant degree of enhancement. The degree of inhibition was always lower than that obtained with polyclonal anti–N-CAM Fab (Fig. 2), but the rate of aggregation could be decreased by as much as 70–80% by combining different inhibitory monoclonal reagents, for example 12B and 5E. The two antibodies directed against carbohydrate determinants (see below) did not affect adhesion (data not shown). However, these antibodies react with several different glycoproteins (unpublished observations) and therefore were not routinely included in analyses involving unfractionated membrane proteins.

Table I. mAb Staining of Intact and Methanol-permeabilized Retinal Cells

	Cultured cells*		Trypsinized cells*	
Antibody	−MeOH	+MeOH	−MeOH	+MeOH
Nonimmune	−	−	−	−
10A	++	±	−	−
11B	++	+	−	−
9A	+++	++	−	−
12B	+++	+++	−	−
PP	+++	+++	−	−
79B	+++	+++	−	−
5E	+++	+++	−	−
105	++	+++	−	−
30B	±	+++	−	+++
4D	−	+	−	+++

Staining appeared as a ring of patches around the cell perimeter and along neuronal processes (see Fig. 3). The relative intensity of the staining is indicated using an excess of antibody; ± refers to staining that was only slightly above background and did not have a patchy distribution.

* Cultured cells have been allowed to re-express surface proteins that were cleaved during dissociation of the retinal tissue using trypsin. Trypsinized cells were stained immediately after the tissue dissociation.

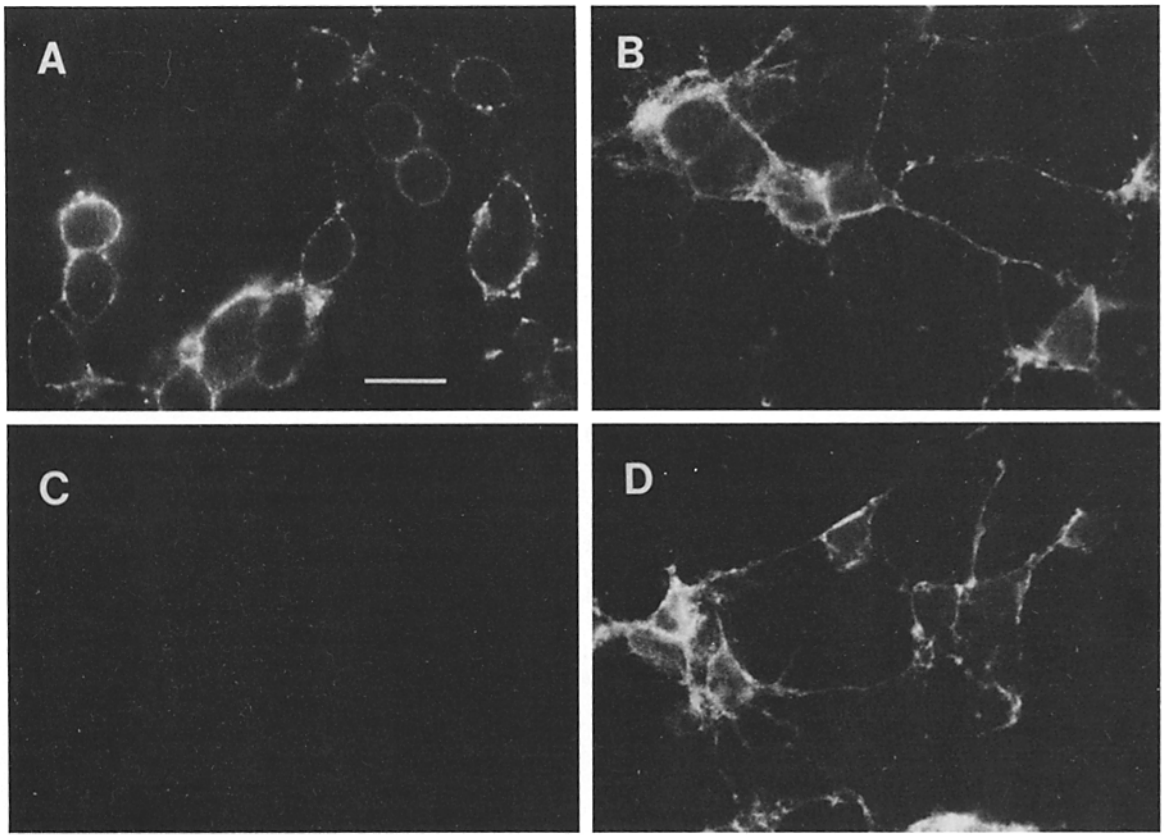

Figure 3. Immunofluorescence detection of mAb epitopes or intact or methanol-permeabilized cells. Cells prepared from embryonic retina were cultured for 2 d and stained by indirect immunofluorescence methods, either live or after treatment with −20°C methanol for 5 min. The mAbs fell into two groups, one group represented here by PP, which stained strongly both without (*A*) and with permeabilization (*B*), and the other group, represented by 4D, which stained permeabilized (*D*) but not intact (*C*) cells. The results obtained for all antibodies tested are summarized in Table I. Bar, (*A*) 10 μm.

Reaction with Extracellular and Cytoplasmic Domains

The antibodies were classified based on their ability to react with extracellular versus membrane-protected or intracellular determinants. Cultured, viable retinal cells showed positive immunofluorescence staining, appearing as a ring of patches around the cell perimeter, with all polypeptide-specific antibodies except 30B and 4D. This extracellular staining was not observed if freshly trypsinized cells were used. Antibodies 30B and 4D did not stain cells with intact membranes, but did stain both cultured and trypsinized cells whose surface membrane had been permeabilized with methanol. Thus 30B and 4D appear to recognize intracellular determinants. Nonimmune Fab did not stain cells under any of the conditions used. These data are summarized in Table I and representative staining is shown in Fig. 3.

Interference among Antibodies in Binding to Brain Membrane Vesicles

Fig. 4 summarizes the competition between pairs of labeled and unlabeled mAb for binding to native, membrane-bound N-CAM. Under the conditions used, each antibody was able to block its own binding by >90% except 105, which completed 75% of its own binding. A different pattern of inhibition was observed for each antibody. Differences in the affinity of two antibodies for the same epitope do not appear to be sufficient to explain the patterns. The results therefore suggest each antibody is directed against a distinct epitope, but do not rule out the possibility that two epitopes share a common structural feature. Only antibody 105 did not affect the binding of other antibodies. In some cases the inhibition between antibodies was reciprocal (9A and 11B, for example); in others (5E and 79B) it was not, suggesting that the relative affinity of one (5E) is higher than the other (79B). Interference was not restricted to antibodies having similar effects on aggregation, as evidenced by the competition between PP (inhibitor of aggregation) and 9A (enhancer).

Recognition of Carbohydrate versus Protein-dependent Determinants

Up to one-third of the weight of N-CAM is carbohydrate, including long chains of polysialic acid and other more typical *N*-linked oligosaccarides (6, 8, 17). The contribution of these carbohydrate moieties to epitopes recognized by the mAbs was assessed by immunostaining dot blots (data not shown) of native N-CAM, neuraminidase-treated N-CAM, and N-CAM digested with endo-F (to remove all N-linked carbohydrate). All of the mAbs stained N-CAM after removal of sialic acid. Although both 104 and CHO failed to recognize N-CAM treated with endo-F, the remaining 10 antibodies continued to react with the residue polypeptide. Thus, two of the monoclonal reagents require the presence of an N-linked, mannose-rich core structure and the other 10 appear to be directed primarily against polypeptide determinants.

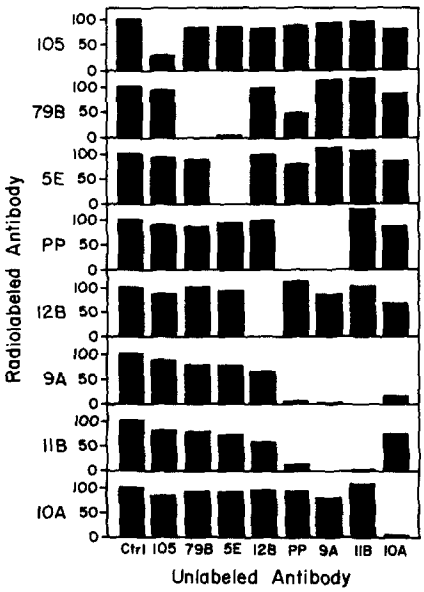

Figure 4. Interference between mAbs in their binding to native N-CAM on brain membrane vesicles. The vesicles were incubated with one unlabeled mAb and then tested for their ability to bind a second ^{125}I-labeled antibody. The values shown represent the percent radiolabel bound relative to controls (ctrl) in which the vesicles were not treated with an unlabeled antibody. Each antibody blocked its own binding, and in some cases the binding of other antibodies. The standard deviations ($n \approx 6$) in the different combinations ranged from 4–7%.

Reactivity of Antibodies with the 65-kD Amino-terminal Fragment

Under appropriate conditions, N-CAM appears to degrade spontaneously, yielding a relatively stable 65-kD fragment that has been shown to contain the amino terminus, cell-binding activity, and only a small amount of the total sialic acid (6). Shown in Fig. 5 is an immunoblot of purified 65-kD fragment that had been run on 15% gels and stained with the indicated antibodies. All antibodies except 4D, 30B, and 105 reacted strongly with this fragment, suggesting that most of the epitopes recognized in our studies are associated with the cell binding domain of the molecule.

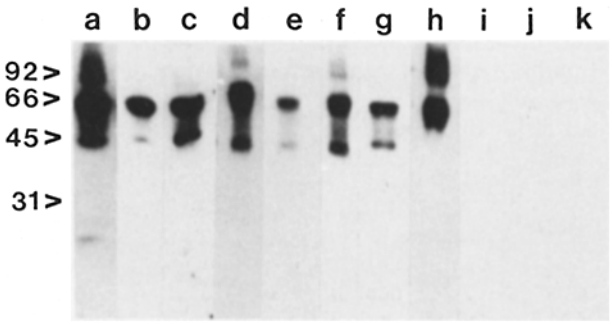

Figure 5. Identification of epitopes associated with the amino-terminal 65-kD fragment of N-CAM (Frl). Partially purified Frl-containing digests (see Materials and Methods) were further fractionated by SDS PAGE and analyzed by immunoblotting with each mAb. Antibodies in lane *a*, 5E; lane *b*, 79B; lane *c*, PP; lane *d*, 12B; lane *e*, 9A; lane *f*, 11B; lane *g*, 10A; and lane *h*, CHO, all stained Frl, whereas lane *i*, 105; lane *j*, 30B; and lane *k*, 4D, did not. Antibody 104 gave the same staining pattern as CHO.

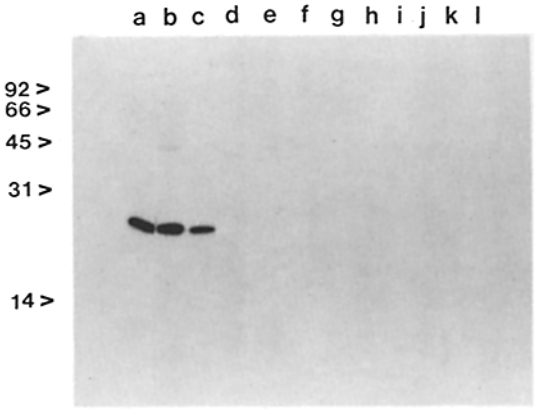

Figure 6. Epitopes associated with the 25-kD heparin-binding fragment. Immunoblot analysis of this fragment after SDS PAGE indicated the presence of epitopes. Antibodies in lane *a*, BlA3 (the mAb described by Cole and Glaser in ref. 3); lane *b*, 5E; and lane *c*, 79B. Antibodies PP (lane *d*), 12B (lane *e*), 9A (lane *f*), 11b (lane *g*), 10A (lane *h*), CHO (lane *i*), 105 (lane *j*), 30B (lane *k*), and 4D (lane *l*) did not recognize this fragment.

Reactivity of Antibodies with the 25-kD Heparin-binding Fragment

Cole and Glasser have recently reported that a 170-kD glycoprotein isolated from adherons and containing a heparin binding site is, in fact, N-CAM (3, 4). They have proposed that, on cells, this binding activity is directed at heparan sulfate that is itself associated with the membrane, and that the interaction of N-CAM with the glycosaminoglycan enhances N-CAM–mediated homophilic adhesion. Furthermore, the heparin-binding activity has been associated with 25-kD subtilisin fragment of the molecule that is uniquely recognized by a mAb BlA3. The ability of our mAbs to recognize the heparin-binding fragment after SDS PAGE is illustrated in Fig. 6. Antibodies 79B and 5E were strongly reactive, whereas the others produced no staining of material of the appropriate electrophoretic mobility.

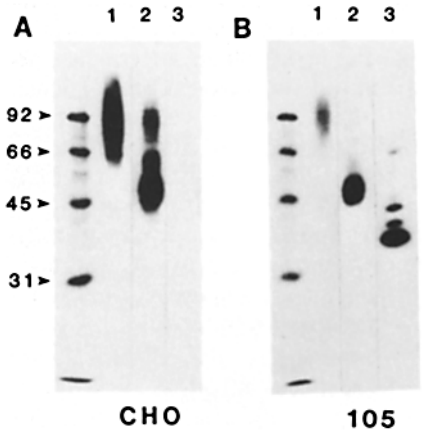

Figure 7. Epitopes associated with the polysialic acid–containing CNBr fragment of N-CAM. In immunoblots, only antibodies CHO (*A*) and 105 (*B*) were found to recognize this fragment. The antibody-stained material appeared in SDS PAGE as a diffuse band (lane *1*). After removal of most polysialic acid with neuraminidase, both antibodies predominantly recognized a band of higher electrophoretic mobility (lane *2*). Removal of all N-linked carbohydrate with endo-F yielded a 36-kD component (lane *3*) that was stained by 105, but not by CHO.

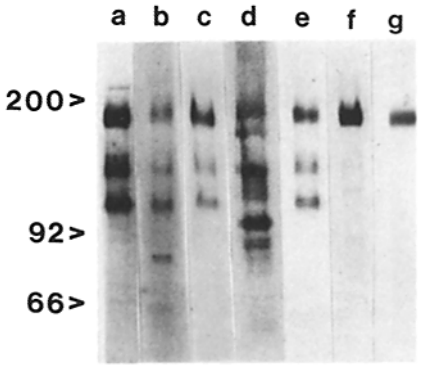

Figure 8. Reactivity of mAbs with the three major polypeptide forms of N-CAM. Detergent extracts of optic nerve, chiasm, and tract of 2.5-wk-old chickens, which contain all three forms, were treated with endo-N, fractionated by SDS PAGE, and analyzed by immunoblotting. Most of the mAbs recognized the three forms as shown here for 5E (lane *a*), PP (lane *b*), 9A (lane *c*), CHO (lane *d*), and 105 (lane *e*). The 104 and CHO antibodies, whose reactivity is dependent on carbohydrate, recognized other glycoproteins in addition to N-CAM, as shown in lane *d* for CHO. Antibodies 30B (lane *f*) and 4D (lane *g*) reacted only with the largest polypeptide form of N-CAM.

Reactivity with a 36-kD Polysialic Acid–containing Glycopeptide

A CNBr fragment of N-CAM containing most of the molecule's polysialic acid was isolated according to the procedures of Crossin et al. (5). Shown in Fig. 7 *A* is the reaction of the carbohydrate-dependent antibody CHO with this fragment. Without glycosidase treatment, this fragment migrated as a diffuse band with an apparent molecular mass range of 60–150 kD. After treatment with endo-N, the electrophoretic mobility of the stained material was increased, and removal of N-linked carbohydrate by endo-F eliminated recognition of the fragment by antibody CHO. Of the other mAbs only 105 stained the untreated and endo-N–treated fragment. In addition, 105 also stained a 36-kD component in the endo-F–treated sample (Fig. 7 *B*), indicating that this antibody recognizes a polypeptide determinant associated with the polysialic acid–containing CNBr fragment.

Reactivity of Antibodies with Different Polypeptide Forms of N-CAM

SDS PAGE immunoblots of detergent extracts from postnatal chick optic nerve, chiasm, and tract reveal the three naturally-occurring N-CAM polypeptides that have been proposed to differ in the length of their carboxyl-terminal domains (6, 12). With our SDS PAGE conditions, the apparent molecular mass of these forms is 180, 140, and 120-kD after removal of their polysialic acid, and 160, 130, and 110-kD after treatment with endo-F. The ability of each mAb to recognize in SDS PAGE immunoblots the three major naturally occurring polypeptide forms of N-CAM is shown in Fig. 8. Antibodies 4D and 30B appear to react only with the 180-kD form, while all other antibodies recognize all three forms. These results suggest that the 4D and 30B epitopes are located within the carboxyl-terminal 30 kD of the 160-kD N-CAM polypeptide.

Discussion

This report describes the identification and characterization of mAbs with different specificities capable of recognizing a variety of functional and structural features of the N-CAM molecule. The implications of these studies for specific N-CAM regions and functions are considered here. The results are discussed with respect to the whole molecule in the accompanying paper.

Of the 12 antibodies studied, two were directed against carbohydrate-dependent epitopes and the remainder against the polypeptide. The carbohydrate-recognizing antibody CHO reacted with both the 65-kD amino-terminal fragment and the 36-kD sialic acid–containing fragment, suggesting, as supported by previous studies (5, 6) and confirmed in the accompanying report (10), that this antibody recognizes more than one site on the molecule. Each of the polypeptide-dependent epitopes did not recognize more than one of the major N-CAM regions (binding, polysialic acid–containing, and cytoplasmic), and the differences in their ability to affect each other's binding suggests that they are directed against distinct determinants.

A notable feature of the 10 anti-polypeptide reagents was that although they were initially selected for the ability to detect fragments of N-CAM in immunoblots, seven reacted with the amino-terminal portion of the molecule and had a direct effect on cell adhesion. All but one of the antibodies against polypeptide-dependent determinants were produced through immunization and selection with purified N-CAM; therefore, the apparent preference of the mAbs for the binding region does not reflect the fact that this region exists outside the cell. Nevertheless, in spite of the abundance of monoclonal reagents that affect N-CAM binding functions, the ability of polyclonal antibodies to inhibit cell–cell aggregation was substantially greater than even combinations of the monoclonals. The reason for this difference is not known, but might reflect a higher affinity of some antibodies in the polyclonal response, or their reaction with an as yet unidentified N-CAM binding site.

The seven antibodies whose Fab fragments affected cell adhesion could be divided into three distinct groups: two that inhibited adhesion and also recognized the 25-kD heparan sulfate–binding domain (5E and 79B), two that inhibited adhesion but did not recognize the 25-kD fragment (PP and 12A), and three that enhanced adhesion (11B, 9A, and 10A). Despite the different activities of the antibodies, the proximity of their epitopes in the native conformation of the molecule is suggested by the fact that pre-exposure of membranes to 9A interfered with the subsequent binding of at least one antibody in each of these groups, and strong interference was also observed within each group.

In contrast, the two carbohydrate-dependent antibodies (CHO and 104) and a third polypeptide-dependent (105) antibody that recognized epitopes in the sialic acid–rich fragment did not have any effect on cell adhesion. These results are consistent with the previous suggestion that the major binding activity of N-CAM is associated with the 65-kD fragment and does not require carbohydrate (6).

In summary, the present studies both expand the repertoire of reagents that mark the binding, polysialic acid–bearing, and cytoplasmic domains of N-CAM (3, 5, 6, 12) and provide new immunochemical probes, most notably epitopes asso-

ciated with the 180-kD form and with antibodies that perturb cell–cell aggregation. As described in the following paper (15), the value of these reagents and the ability to relate them to antibodies and studies from other laboratories has also been enhanced by the use of peptide fragment mapping techniques to place the epitopes in the overall structure of the molecule.

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