Cell Adhesion Molecules

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Summary. It has been proposed that cell-cell recognition occurs by means of local cell surface modulation of a small number of proteins rather than by expression of large numbers of different cell surface markers. Several different cell adhesion molecules (CAM's) have now been found in a number of vertebrate species in different tissues such as liver and striated muscle and even in a single complex structure such as the brain, where different molecules specific for neurons and glia have been identified. The neuron-specific molecule is involved in early embryonic events but also mediates neurite fasciculation, neuromuscular interaction, and orderly layering of neural tissue. It undergoes local surface modulation with loss of sialic acid during development. A failure of this process is closely correlated with connectional disorders in the *staggerer* mutant of the mouse. The accumulated data on this and other CAM's favor modulation theories rather than strict chemoaffinity theories of cell-cell recognition.

There is at present no adequate general theory of development, at least in the same sense that there are adequate theories of evolution and genetics. This is perhaps not surprising: the microscopic nature of developmental primary processes and the occurrence of parallel yet coordinated events linking these processes are particularly strong examples of biological complexity. Each of these primary processes (*1*)—cell division, migration, cell recognition and adhesion, differentiation, and cell death—defies simple analysis in terms of lists of genes or gene products and their linear interactions. Nevertheless, we still need a much clearer description of these processes at the molecular level before any general theory of development can be attempted.

Cell-cell adhesion is likely to be the first primary process to be understood at the molecular level. Because direct molecular binding is the central event in adhesion, the isolation of cell-cell adhesion molecules should allow a physicochemical analysis of their major properties in the absence of the larger cellular system in which they function. About 8 years ago, I began to study the possibilities that local cell surface modulation (*2*) might be a key factor in cell-cell adhesion and that new techniques for eliciting highly specific antibodies to the cell surface could be instrumental in its analysis. A year later, I had the good fortune to be joined by several young investigators who shared the conviction that this approach to cell-cell recognition would be successful in identification of the molecules involved, ignorance about which was a major deadlock in the field.

The program adopted was to develop an assay for adhesion analyzable in molecular terms, to obtain specific antibodies to adhesion molecules, to search for modulation of the structure and function of these molecules at the cell surface (*2*), and finally to interpret the binding behavior of these molecules in terms of interactions with other primary processes in development. In this article, I discuss some conclusions and hypotheses that have emerged from this ongoing work as well as from related recent efforts in other laboratories. I consider mainly cell-cell adhesion in vertebrates, emphasizing its role in histogenesis and paying particular (but not exclusive) attention to the nervous system [see (*3*) for nomenclature].

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Theories of Adhesion

A crude discrimination can be made among the kinds of theories that have been proposed to account for cell adhesion. The first kind ascribes the cellular binding and recognition events within structures even of a single tissue to different surface marker molecules. An example is Sperry's chemoaffinity hypothesis (*4*), proposed originally to account for the exquisite mappings of neural cells in the central nervous system. In its most rigorous form, such a theory requires a multiplicity of different gene products whose bindings are pairwise complementary from cell to cell. In contrast, modulation theories (*2*) assert that, in general, tissues will have only a few cell-cell adhesion molecules (CAM's), corresponding in number perhaps only to the number of major classes of cells and tissues. Pattern would arise either from alterations in the temporal expression of these molecules or from other forms of local surface modulation (*2*), for example, a chemical alteration of these molecules that results in a change in their binding properties. Modulation theories are compatible with more noncommittal phenomenological or thermodynamic theories of adhesion (*5*) but are not coextensive with them. Finally, another group of theories (*6*) asserts that adhesion takes place via weak forces such as surface charge and van der Waals interactions, but not as identified with a specific gene product or particular surface macromolecule. Sufficient evidence already exists to suggest that such theories are no longer tenable. The issue is therefore to decide between chemoaffinity theories and modulation theories.

Chemoaffinity theories require different highly specific complementary interactions among their postulated surface markers, insofar as they assert that major histogenetic patterning and mapping are largely determined by the variety and specificity of such markers. Modulation theories require specificity within a tissue system and between a few cell types in that system but have lesser requirements for a range of specificities insofar as they ascribe histogenetic patterning to a complex of processes acting on a few kinds of CAM. Thus, the empirical burden is to show the existence of a great variety of surface markers or of only a relatively small number of tissue–system specific CAM's subject to processes of modulation. One of the major tasks of this article is to provide evidence to support modulation theories and, in the case of the central nervous system at least, to rule out strict chemoaffinity theories.

Assays Suitable for CAM Identification

For CAM identification, an initial distinction must be made between specific binding between identifiable single cells and aggregation of large numbers of cells

(*7, 8*). A review of various methods has been given (*3*); here, my purpose is to sharpen the issue around the goals of CAM isolation. The adequate conditions and pragmatic limits of such isolation procedures are illustrated in Fig. 1. The main aim is to arrange conditions as much as possible to resemble molecular binding, that is, as if the ligands could be tested free of their parent cells. This is not a completely realizable goal because of the inability to define equilibrium conditions and because of the strong kinetic constraints on cell-cell binding.

The necessity of dissociating cells from their parent tissues, sometimes by radical means such as enzymatic cleavage, requires reformation of cell surface receptors as demonstrated by the presence of a normal cell surface map (*7*). Moreover, different portions of highly complex tissues such as the brain may develop on different schedules and heterologous assays between cells from these regions may show appearances of false specificities. The solution is to test all age epochs under homologous and heterologous cell-binding conditions. At best, a CAM assay is only an initial screen to be checked by subsequent analyses at each stage of CAM isolation and purification. This task is greatly simplified by the use of specific immunization techniques.

Immunological Identification and Isolation of CAM's

Antibodies against adhesion molecules were first used by Gerisch and his coworkers (*9*) in studies of slime mold aggregation. My colleagues and I have used a similar approach in studies of CAM's from embryonic tissues, but in addition found it necessary to devise iterative immunization techniques as well as to use monoclonal antibody methods in an effort to obtain the most specific reagents possible.

Antigen fractionation and iterative immunization. The basis of this method is shown in Fig. 2 in which N-CAM is used as an example. This iterative approach was used successfully to produce serum having a high degree of specificity to N-CAM (*8*). The efficacy of this procedure can be seen in Fig. 3, in which cell surface maps made with the initial antiserum and the antiserum from iterative immunization are compared.

Monoclonal antibodies to CAM. With the use of standard procedures, hybridoma cells secreting monoclonal antibodies have been raised against chicken N-CAM (*10*). One of the clones, 15G8, produced antibodies specific for the sugar moiety of chick N-CAM, as indicated by the inhibition of binding by sialic acid and the failure to bind to N-CAM treated with neuraminidase. This antibody cross-reacted with N-CAM in mouse brain, and the mouse antigen could be purified by affinity chromatography on Sepharose CL-2B derivatized with 15G8 antibody. After denaturation by boiling in 2 percent sodium dodecyl sulfate (SDS) this mouse antigen was then used to raise other mouse monoclonal antibodies. A particular clone, 9E11, produced antibodies which reacted with mouse N-CAM and which appeared to be directed to the polypeptide determinants or possibly sugars other than sialic acid.

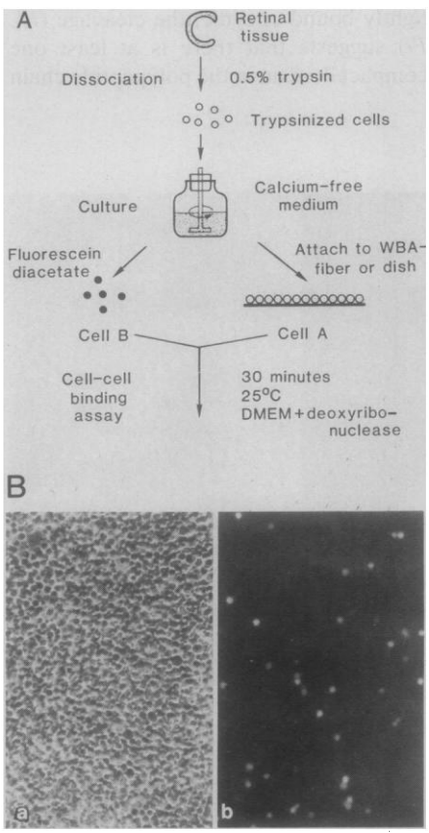

Fig. 1. Procedure for two-cell assay of adhesion. The assay should be short term and single identifiable cells from a known developmental age epoch whose surface pattern demonstrably resembles that of cells in the organ should be used. In addition, the assay should reflect reproducible cell numbers, densities, collision rates, and shear forces, and be clearly definable in terms of possible medium effects, cofactors, cell viability, and metabolic states. (A) After culture to allow regeneration of cell surface proteins, cells are divided into two portions. One is labeled with fluorescein diacetate; the other is attached to a surface with wax bean agglutinin (*WBA*). The cells are shaken for 30 minutes at defined shear force in DMEM (Dulbecco's modified Eagle's medium) and in the presence of deoxyribonuclease to destroy DNA, which induces artifactual aggregation. (B) The assay is read by fluorescence microscopy after unbound cells are washed away. (a) Phase-contrast microscopy; (b) fluorescent-bound cells.

The monoclonal antibodies could be used in affinity chromatography to purify mouse antigens and N-CAM antigens from other species. N-CAM antigens purified on such affinity columns could themselves be bound to Sepharose CL-2B and used further to purify by affinity methods the heterogeneous antibodies raised by iterative immunization in rabbits. This combination of iterative immunization, monoclonal antibody methods, and affinity methods provides a powerful armamentarium for isolating CAM's, both across species lines and in exploring new tissues.

So far, these methods have been used to identify or isolate N-CAM's of different animal species from retina, brain (*7, 8, 10, 11*), and muscle (*12*); L-CAM (*13*) from chick and rat liver; and neural GN-CAM, mediating binding of astrocyte-like glial cells with neurons (*14*). By similar methods, a calcium-dependent trypsin-resistant molecule with little or no organ specificity has been tentatively identified (*15*), and an early protein related to compaction in morulas has been found (*16*). So far, however, only N-CAM has been characterized sufficiently to relate structure to function.

Chemical and Structural Properties of N-CAM

The N-CAM's are large sialoglycoproteins with several unusual properties; they are found mainly on the cell surfaces of neurons and striated muscle precursors (myoblasts and myotubes). They do not appear to be present on glial cells. Most of the chemical studies (*17, 18*) have been carried out on chick N-CAM and, unless otherwise specified, the description given here applies to that species. In general, however, there are strong structural and functional resemblances among N-CAM's from different species (*10, 11*).

N-CAM appears to be an integral membrane protein, as indicated by the need for detergent to extract it from membrane preparations. Intrinsic labeling experiments indicate that both the protein and carbohydrate portions are synthesized in the cell of origin. The molecule is a single polypeptide chain with a molecular weight of 120,000 with additional variable amounts of carbohydrate, that shows self-aggregation and high intrinsic viscosity in aqueous solutions. N-CAM also shows microheterogeneity even in the monomer: after SDS electrophoresis, N-CAM from embryos

migrates as a broad continuously stained region spanning an apparent molecular weight of 200,000 to a molecular weight of 250,000 (Fig. 4A).

The microheterogeneity of embryonic N-CAM is mainly attributable to its carbohydrate portions which are unusual in both composition and structure. Embryonic N-CAM contains at least 26 to 35 percent carbohydrate, of which almost four-fifths is sialic acid (*17*); unlike sialic acid, other sugars are present in amounts comparable to those in many glycoproteins. The sialic acid appears to be covalently linked but is only slowly released from the protein by either neuraminidase or acid, suggesting an unusual linkage of sialic acid to protein either directly or via other sugars. Neuraminidase treatment removes 99 percent of the sialic acid (*17, 18*) and afterward N-CAM appears as a closely spaced doublet with an apparent molecular weight of 140,000 (Fig. 4B). The amino acid composition (*17*) of N-CAM is not unusual, and analysis of proteolytic fragments suggests that all forms of the glycoprotein are derived from similar polypeptide chains. The heterogeneity of N-CAM is thus largely the result of variations in sialic acid.

Even in the most purified N-CAM preparations, exposure at 37°C to low salt buffer induces spontaneous proteolytic cleavage to a polypeptide with a molecular weight of 65,000 plus smaller peptides (Fig. 4C). The cleavage does not occur in 0.5*M* sodium chloride. Whether it is the result of intrinsic proteolytic activity or of a contaminating tightly bound enzyme, the cleavage (*17, 19*) suggests that there is at least one compact domain in the polypeptide chain followed by an exposed stretch of loosely folded chain. Preliminary evidence (*20*) suggests that this entire region includes the amino terminus and that it comprises part of the binding domain.

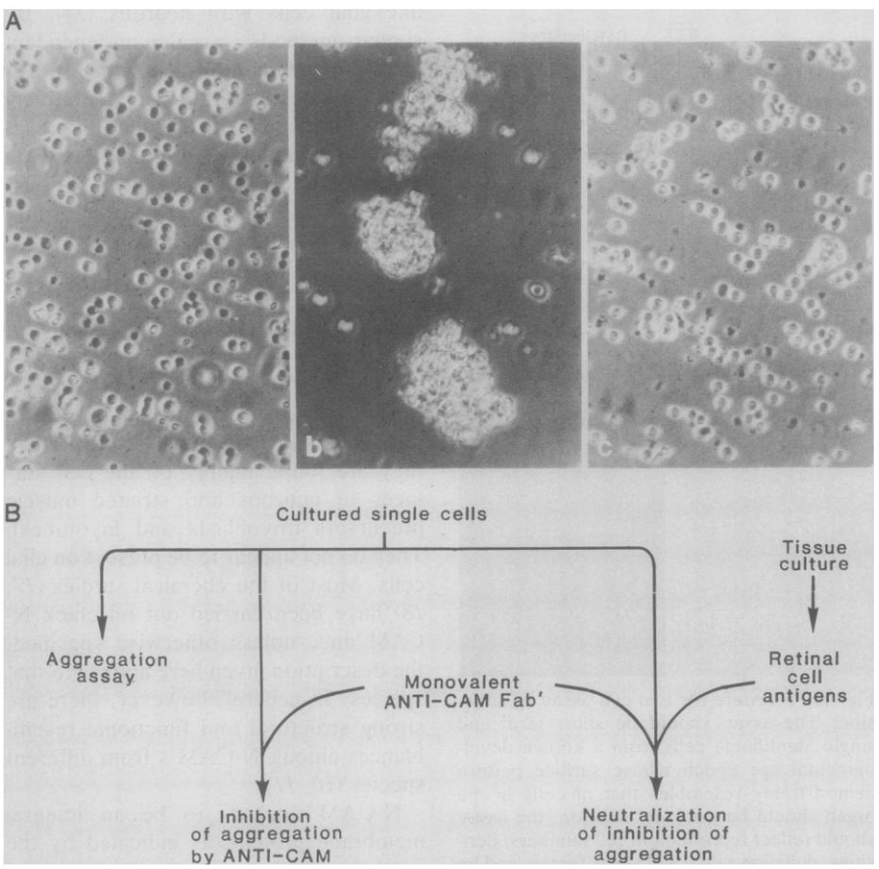

Fig. 2. Inhibition of aggregation by anti-N-CAM Fab' fragments and a neutralization assay procedure based on this phenomenon. Two-cell assays (Fig. 1) are first correlated with assays measuring the decrease of single chick brain or neural retinal cells accompanying aggregation in shaken suspensions. If good correlation is obtained, the disappearance-of-single-cell assay (DSC assay) is routinely employed. Rabbits are immunized, for example, with 10-day-old chick retinal cells, and Fab' fragments are prepared from the immunoglobulins in the immune serums. The fragments are then screened for those that inhibit the DSC assay. Tissue culture supernatants from neural retinal cells are fractionated, and the fractions are screened for ability to neutralize the inhibition by Fab' fragments obtained from active serums. The neutralizing fractions are then used to immunize other rabbits in order to produce further inhibitory serum presumably of increased specificity. If necessary, the procedure is repeated again. (A) Dissociated retina cells prior to aggregation (a), cell aggregates after shaking for 30 minutes at 37°C (b), and cells shaken for 30 minutes at 37°C in presence of Fab' fragments (c). (B) Neutralization procedure; appropriate retinal cell antigens mixed with anti-N-CAM Fab' fragments neutralize the inhibition.

Binding Properties of N-CAM

Because of a tendency toward self-aggregation in aqueous solution, the binding mechanism and binding strength of N-CAM are difficult to determine quantitatively. Comparison of the effects on binding of free N-CAM as well as of lipid vesicles containing only N-CAM, however, permits some conclusions about the mechanism to be drawn (*21*). Artificial lipid vesicles containing N-CAM became bound to various cell types with a specificity comparable to that of neurons. Soluble N-CAM bound in a similar fashion after a brief exposure to *p*H 3 and, in both cases, Fab' fragments of antibody to N-CAM (anti-N-Cam) blocked the binding. Neither kind of binding required calcium.

These studies suggest that N-CAM binding is second-order homophilic (*3*). Perhaps the strongest evidence to support this conclusion was obtained from experiments (*21*) in which prior coating of a cell with Fab' fragments of a monoclonal anti-N-CAM strongly inhibited binding of N-CAM, N-CAM vesicles, or other cells having N-CAM, despite the fact that these ligands had not been exposed to the Fab' fragments. Neuraminidase treatment of N-CAM does not alter the binding; indeed, neuraminidase-treated free N-CAM appears to bind even more effectively to cells than native embryonic N-CAM. These findings indicate that sialic acid is not directly involved in the binding and are consistent with the possibility that the sialic acid–free fragment with a molecular weight of 65,000 contains at least some of the binding regions of the molecule.

Conversion of Embryonic to Adult N-CAM in vivo

Apart from the limited proteolysis of N-CAM, which has been observed only in vitro, the chemical property of most functional significance for modulation theories is the conversion in vivo of the molecule from the embryonic (E) to several adult (A) forms (*18*). Embryonic N-CAM has a sialic acid content of up to 26 percent by weight, whereas the adult forms contain only about 9 percent of this sugar. In contrast, the amino acid composition, and the contents of amino

sugars and neutral sugars of the E form and the A forms are strikingly similar. Furthermore, sequence analysis, comparisons of CNBr fragments, and peptide maps all suggest that the polypeptide chains are closely similar if not identical.

The A forms can be recognized on sodium dodecyl sulfate electrophoresis as three relatively sharp bands with apparent molecular weights of 180,000, 140,000 to 150,000, and 120,000 (an example is shown later in Fig. 7). In most cases, some E form is also present in much smaller amounts. The A forms are not identical to neuraminidase-treated E or A forms, both of which give a doublet having a molecular weight of 140,000. Nonetheless, the data suggest the conclusion that the E → A conversion consists either in failure synthetically to attach as much sialic acid with increasing maturation or the removal of more than half of the sialic acid by surface sialidases during development.

The E → A conversion represents a clear-cut example of the local surface modulation that had been predicted (*2*) to occur in cell recognition events. Conversion takes place at different times and rates in different portions of the brain; in mice, much of the conversion is accomplished in the early perinatal period. It is not known whether conversion occurs uniformly for every molecule on a given cell or reflects an average value for different cell populations; but it is known that E forms of brain, retina, and muscle have somewhat different mobilities on gels. Clarification of the cellular mechanism of the conversion may have great significance for modulation theories of adhesion. To illustrate how this might be so, a tentative binding model of N-CAM based on the available chemical facts may be useful. Of course, this model is highly schematic, is full of gaps, and is subject to alteration as more precise structural data are obtained.

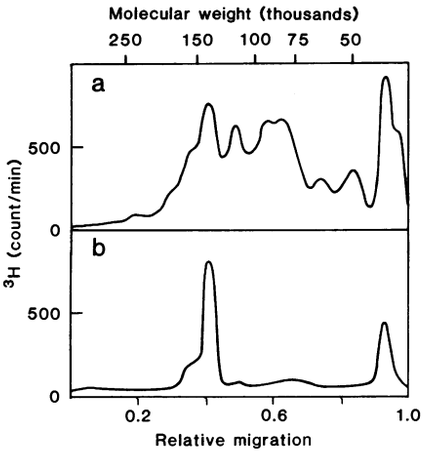

Fig. 3. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of immunoprecipitated ^{3}H-labeled proteins extracted from membranes of retinal cells from 10-day-old chick embryos. (a) Immunoprecipitation with rabbit antibodies to whole retinal cells (the complexity of the membrane protein population is evident). (b) Immunoprecipitation with antibodies to purified activity after iterative immunization. Because the cells were cultured in suspension, the major peak probably corresponds to N-CAM largely lacking sialic acid (*17*). The peak showing a molecular weight of about 42,000 probably represents actin, which was nonspecifically precipitated.

Hypothetical Binding Model of N-CAM

The key facts related to N-CAM binding are the following. (i) The binding is second-order homophilic. (ii) Preliminary observations (*20*) suggest that the NH_2-terminal 65,000 molecular weight fragment inhibits binding of N-CAM to cells and neutralizes inhibition of binding by anti-N-CAM. (iii) Binding is not eliminated by removal of sialic acid; indeed, there are strong indications that it may be enhanced. (iv) E → A conversion occurs at different rates in different parts of the brain in the perinatal period, but is never complete even in the adult brain. (v) The E form is microheterogeneous in sialic acid; this may provide a basis for diversity in binding strengths necessary during development. The binding model shown in Fig. 5 takes these facts into account.

The key assumption of this charge-perturbation model is that the homophilic binding is reduced in free energy by the very high charge density of the sialic acid linked to the molecule in a region or regions adjacent to the binding region. This occurs either as a result of electrostatic repulsion between the sialic acid (possibly present as polysialic acid) and the 65,000-dalton domain of the molecule from the opposite cell, by mutual repulsion of the two sialic acid–rich regions of each of the N-CAM's, or by induced alterations in the conformation of the binding regions that do not contain sialic acid. Whatever mechanism obtains, E → A conversion would lead to a strong increase in the free energy of binding. This model takes account of the fact that N-CAM represents about 1 percent of all brain cell surface proteins (*17*) and that it is mobile (*22*) in the plane of the membrane (diffusion constant, $D = 6 \times 10^{-10}$ square centimeters per second). It also recognizes the possibility that the regions of the molecule rich in sialic acid may govern the spacing of CAM's or the polymeric combination of chains on the same cell, with subsequent alterations in binding after conversion. A strong prediction of the model is that there will be a monotonically increasing order of N-CAM to N-CAM binding strengths: E-E < E-A < A-A < neuraminidase treated–neuraminidase treated.

Undoubtedly, much of this model will have to be revised with new structural information. For example, it is not ruled out that protein from one N-CAM binds to non–sialic acid carbohydrate from the opposing N-CAM molecule. A particularly attractive feature of this minimal model is that, in view of the microhetero-

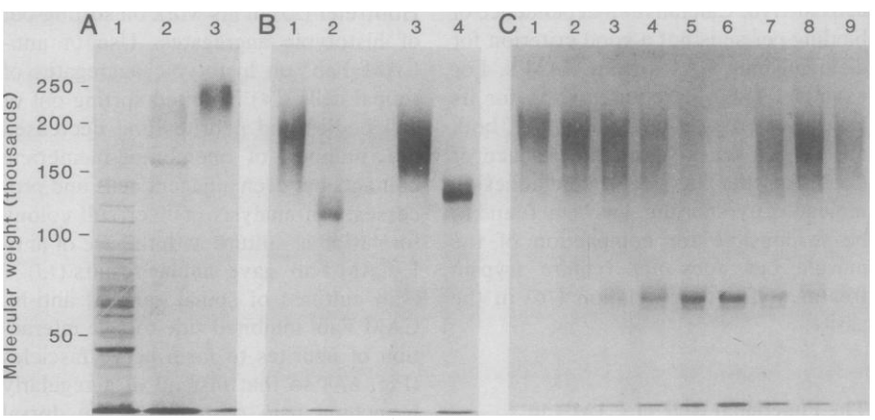

Fig. 4. (A) Gel electrophoretic comparisons of chick N-CAM. (Lane 1) Nonidet NP-40 extract of embryonic chick brain membranes, (lane 2) N-CAM purified by chromatography, (lane 3) N-CAM purified by affinity chromatography on monoclonal antibody columns. (B) Effect of neuraminidase treatment. (Lane 1) N-CAM, (lane 2) neuraminidase-treated N-CAM, (lane 3) reduced N-CAM, (lane 4) reduced neuraminidase-treated N-CAM. (C) Autolysis products of N-CAM incubated at 37°C, *p*H 8.3. (Lane 1) No incubation, (lane 2) incubation for 2 hours, (lane 3) incubation for 6 hours, (lane 4) incubation for 24 hours, (lane 5) incubation for 48 hours, (lane 6) incubation for 96 hours, (lane 7) incubation for 96 hours at 25°C, (lane 8) incubation for 96 hours at 4°C, (lane 9) incubation for 96 hours at 37°C plus 0.5*M* NaCl. Molecular weight numbers refer to (A) only; other gels have higher values displaced downward by 25,000. All figures refer to apparent molecular weights.

geneity introduced by sialic acid, it provides a graded means for the generation of a diversity of affinity differences in a single binding molecule that is general for the nervous system. The most striking biological support for this local surface modulation model is evidence, to be reviewed below, that regional failure of E → A conversion is present in certain mouse cerebellar mutants.

Ion Dependence and Stages of Adhesion

Independent of modulation mechanisms, which perforce must invoke at least two successive stages, a case may be made for several stages in the relatively immediate process of cell-cell adhesion. Such ideas have provided background for the identification of a set of molecules (*15*) that appear to be distinct from cell-specific CAM's and that mediate relatively nonspecific adhesion among cells of the same and different organs. To demonstrate the existence of such molecules, the cells were treated with trypsin in the presence of Ca^{2+}. This treatment effectively destroys CAM molecules and other surface molecules, but leaves the trypsin-resistant cell adhesion molecules or CAT's (CAT, cell adhesion molecule, trypsin-resistant).

CAT's are not only resistant to trypsin in the presence of Ca^{2+} but require the ion for their binding mechanisms. The known CAM's and CAT's are not immunologically cross-reactive (*15*). It remains unclear whether these two systems of molecules can act simultaneously or successively in cells that have not been treated with trypsin, for example, cells in vivo. Calcium ion–dependence of binding per se is not a good criterion for distinguishing CAT's from CAM's. For example, L-CAM requires Ca^{2+} for its action, whereas N-CAM does not; both are trypsin-sensitive in the absence of Ca^{2+}. Another Ca^{2+}-dependent adhesion molecule, uvomorulin, has been found to be responsible for compaction of the morula but does not require trypsin treatment for its revelation (*16*) in the assay.

The Functional Role of CAM's in Cell-Cell Interactions in vitro

A number of studies have been carried out on in vitro functions of the N-CAM and L-CAM systems. One of the striking findings derived from such studies was that N-CAM was present on all neuronal cells and their processes regardless of their morphology or differentiation state.

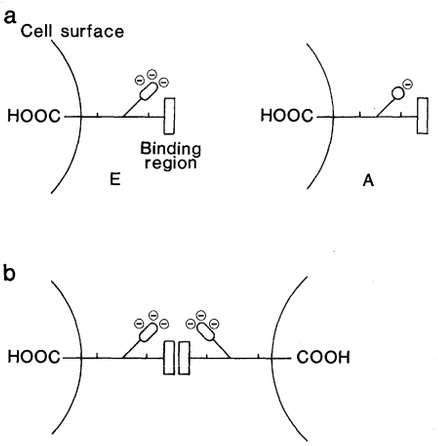

Fig. 5. Schematic charge-perturbation binding model for N-CAM. (a) E and A forms. The sialic acid–free binding region is represented by the rectangle and the sialic acid–rich sugar moeity by an oval; the possibility of more than one site of sugar attachment is shown by short vertical lines. The E → A conversion removes large amounts of sialic acid. (b) Second-order homophilic binding. Free energy of binding is assumed to be perturbed by the negatively charged region via repulsion, conformational change or redistribution of N-CAM's on cell surface. The strong prediction of the model is that E → A conversion leads to an increase in free energy of binding. This is a binding model; it is not intended to be accurate or representative of such important molecular structural features as the number of chains per binding unit (and therefore the valence) or of the exact location and structure of the sugar-bearing regions.

Moreover, N-CAM was present on neuronal plasma membranes regardless of whether they were in apposition to those of other neuronal cells.

The classic analyses in vitro of the relation of tissue patterning to adhesion and mobility of cells were carried out by Holftreter (*23*) in his work on sorting-out of histotypic aggregates. Use of anti-CAM Fab′ on histotypic aggregates of retinal cells (*24*) inhibited sorting out of cell bodies and neurites and decreased the number of membrane-membrane contacts between adjacent cells and processes. An analysis of liver cell colony formation in culture with the use of anti-L-CAM Fab′ gave similar results (*13*).

In cultures of spinal ganglia, anti-N-CAM Fab′ inhibited side-to-side interaction of neurites to form nerve fascicles (Fig. 6A) so that instead of a regularly branching halo of bundles, the dorsal root ganglion in culture was surrounded by a tangled net of fine neurite processes in a "spaghetti-like" pattern (*25*). Gradients of nerve growth factor (NGF) cause directional growth of neurites from a ganglion (*26*). Anti-CAM Fab′ fragments dramatically reduced this anisotropy (*27*). These findings suggest that side-to-side adhesive interactions among a constant number of neurites may influence the guidance of nerve bundles by amplifying and sustaining the results of a directional signal that is initially followed by pioneering individual neurites and growth cones.

Similar, but not identical, effects of pioneering neurites were seen in investigations (*12, 28*) of nerve-muscle interaction in vitro mediated by N-CAM. Cinematographic studies of chick spinal cord explants in culture with myotubes showed that neurites and fascicles grew to the myotubes and immediately turned to grow down these structures. Anti-N-CAM Fab′ fragments completely obliterated this process; indeed, in the absence of other cells, stable neurite extension from the spinal cord explants was sharply curtailed. Although these events have not yet been related to synapse formation, N-CAM interactions may help constrain the patching of acetylcholine receptors in the muscle membrane, providing for early synapse formation at the site of neuromuscular contact.

The effects of anti-N-CAM on a wide variety of cell types were shown by analyzing chick embryonic neural retinae placed at 6 days into organ culture (*29*). Retinae cultured in the presence of anti-N-CAM Fab′ had vestiges of identifiable layers, but they were disrupted, lacked sharp boundaries, and showed invasion of cells (particularly ganglion cells) into plexiform layers (Fig. 6B). Although individual cells in anti-N-CAM treated retinae were morphologically similar to those in control retinae, there were few areas of cell-cell contact and membrane apposition as well as large areas of extracellular space.

All these in vitro studies suggest that cell-cell interactions mediated by N-CAM are important in achieving the appropriate arrangement of cells in nervous tissue. But the most impressive support for modulation theories and for the role of CAM's in development has come from in vivo studies of mutants in histogenesis.

In vivo Modulation of N-CAM and Genetic Defects in Brain Histogenesis

A number of mouse mutants with cerebellar defects of cell number, type, location, and connectivity have been discovered (*30, 31*). Of these, the so-called granuloprival mutants, *staggerer* (*sg/sg*), *weaver* (*wv/wv*), and *reeler* (*rl/rl*) have been analyzed. Because these are recessive mutants in histogenesis that might involve failure in control of key enzymes, they appear to be ideal candi-

dates to test the modulation hypothesis. This surmise was stimulated by finding that the E → A conversion in N-CAM occurred at different rates in different parts of the normal brain and by the observation that adult N-CAM and neuraminidase-treated N-CAM both still bound to neurons. Moreover, a number of previous observations in other laboratories on *sg/sg* cells suggested that they retained cell surface properties characteristic of the embryonic state. Wheat germ agglutinin (with weak specificity for sialic acid) agglutinates normal embryonic and postnatal *sg/sg* cells but not normal postnatal cells (*32*). Furthermore, antibodies to sialic acid are bound by normal embryonic and postnatal *sg/sg* cells but not by normal postnatal cells (*33*).

Aside from the loss of granule cells, *staggerer* has a failure in development of mature tertiary dendriditic spines on Purkinje cells and also a failure of synapse formation between these cells and parallel fibers. The charge perturbation model for modulation of N-CAM binding and the E → A conversion suggested the hypothesis that *staggerer*, which unlike *weaver* and *reeler*, does not have glial anomalies (*30, 31*), would be the most likely to have alterations in N-CAM modulation. Experimental tests of this hypothesis were striking and clear-cut (*34*). Unlike normal animals, *sg/sg* animals showed persistence at 21 days postnatal age of diffusely migrating embryonic N-CAM particularly in the cerebellum (Fig. 7). At 21 days, brains of littermates of these *sg/sg* animals showed only slightly delayed E → A conversion as did the cerebral cortex of *sg/sg* animals. The E → A conversion of the cortex and cerebellum of *wv/wv* and *rl/rl* resembled the normal or wild type.

One of the main roles of the E → A conversion may be to arrest otherwise normal processes of axon and dendritic migration and interaction at just the proper time, providing a kind of punctuation mark for certain of the other primary development processes (*35*). This would be the expected result of normal E → A conversion which, according to the charge perturbation model would lead to an increase in the free energy of homophilic binding. Conversion arrest or delay as found in *staggerer* should therefore result in abnormal persistence of migration as well as failure in the maintenance of selected connections at the appropriate time. Although the exact mechanism and causal order relating the genetic defect in *staggerer* to the morphologic defects and to the failed E → A conversion remain to be worked out, the observations made so far support the surface modulation hypothesis (*2*). An attractive auxiliary hypothesis is that many of the failures of connection may be the result of failure of a surface sialidase or an intracellular transferase to provoke E → A conversion at the proper time. Such a failure may in fact turn out to be a major etiologic factor resulting from the *sg* mutation.

While this work on cerebellar mutants suggests a major role for CAM's in histogenesis and patterning, concurrent studies on N-CAM in early embryos have indicated just as fundamental a role for these molecules in early interacting systems of development (*1*).

Cell-Surface Modulation and Cell-Substrate Modulation in Embryogenesis

It would be too narrow a view of histogenesis to consider that form and pattern arise solely from any one mechanism or class of mechanisms such as those considered here for CAM's. Indeed, there is already evidence for three separate kinds of supramolecular systems mediating cellular interactions: cell-cell adhesion mediated by CAM's, cell-substrate adhesion mediated by SAM's [substrate adhesion molecules such as fibronectin, collagen, or laminin (*36*)], and cell contacts via intercellular junctions such as gap and tight junctions and desmosomes (*37*). Each system contains different gene products, but certain portions of each of these systems may interact heterarchically with the others and mutually modulate their functions.

In no case so far have all three systems been examined within a single tissue in terms of formation, temporal appearance, and molecular interactions. A striking example of CAM-SAM interaction is, however, provided by the conjugate relation between N-CAM expression and fibronectin appearance (*38, 39*) in the development of neural crest cells. When crest cells first appear at the dorsal border of the neural tube, they have N-CAM at their surface. In short order, they are surrounded by a fibronectin-rich matrix and begin to move and divide, and the N-CAM at their surface is great-

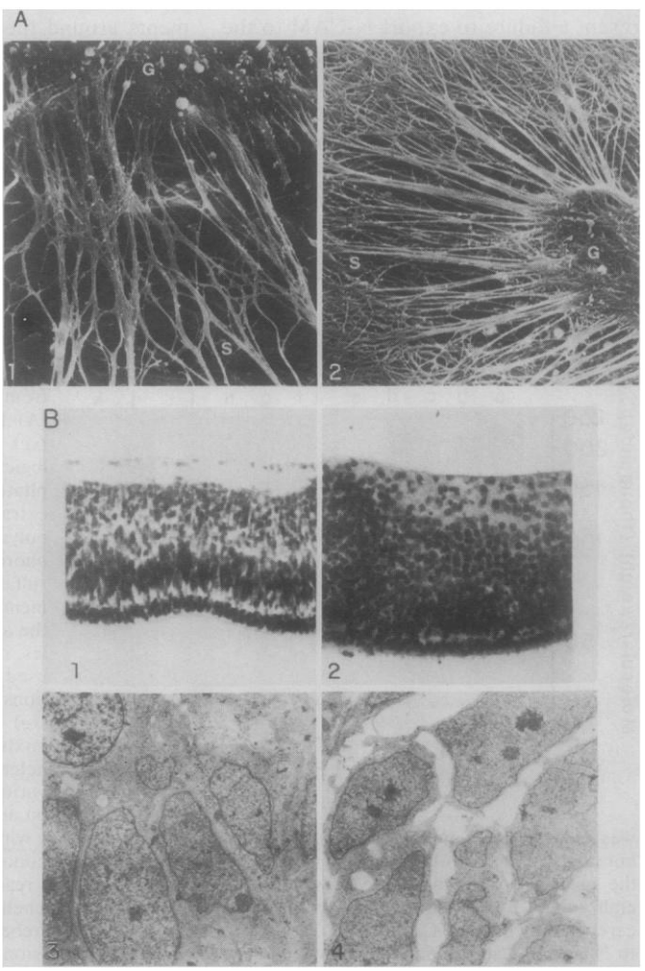

Fig. 6. In vitro activity of anti-N-CAM Fab′ fragments. (A) Fascicles passing from dorsal root ganglion (*G*) of embryonic chick to substratum (*S*) of tissue culture dish (A1) in presence of Fab′ from normal rabbits and (A2) Fab′ from anti-N-CAM. (A1) Thick relatively straight fascicles; (A2) "spaghetti-like" pattern of individual neurites on substrate (× 500). Although growth cones stained for N-CAM, growth cone functions such as neurite elongation, mobility, and substrate attachment were not altered by anti-N-CAM Fab′ fragments. (B) Chick neural retina in organ culture for 3 days after removal from embryo on day 6; (B1) culture in presence of Fab′ from normal rabbit immunoglobulin G; there is normal layered structure (× 650); (B2) culture with anti-N-CAM Fab′ with evident disruption (× 650); (B3) electron micrograph of cells from (B1) (× 5800), showing close apposition of membranes; (B4) electron micrograph of cells from (B2), showing large areas of extracellular space and few areas of cell-cell contact.

Table 1. Early embryonic regions bearing N-CAM (*38, 39*).

Area	Dynamics	Comments
Neural plate and tube	Stable	Present up to formation of adult central nervous system
Notochord	Transient	Areas concerned with induction of primary developmental axis
Somites	Transient	
Neural crest cells	Transient (on; off; on)	Present before migration, absent during migration; present during ganglion formation*
Placodes (optic, otic, pharyngeal)	Transient	Present in tissues to be induced, not in inductor tissues or mesenchyme
Cardiac mesoderm	Transient	
Limb buds	Transient	
Mesonephric tubule precursor	Transient	Appears in proximal precursor, disappears, then appears in distal precursor

*Changes are conjugate with appearance and disappearance of fibronectin.

ly diminished or disappears. On reaching their sites of accumulation, the fibronectin in the surrounding spaces diminishes, N-CAM reappears, and the cells clump to form ganglia. While the appearance and diminution of N-CAM on the cell surface could represent a true regulation of gene expression, it could as well represent a failure to export N-CAM to the cell surface in the course of rapid division. These studies indicate that other gross structural factors and the modulation of SAM's such as fibronectin are also essential for correct directional migration (*38, 39*).

In early embryos, N-CAM appears and remains in regions where it might be expected, such as the neural plate and tube (*38*). But N-CAM also appears and disappears in a number of regions where it was not anticipated to be present. These are regions in which inductive events (*1*) are known to occur, including the notochord, various placodes such as the lens placode, the apical ridge of the limb bud, and the mesonephric rudiments around the Wolffian ducts of the developing kidney. The early picture of N-CAM staining is very dynamic, with appearances and disappearances in these regions in a defined sequence over short periods of developmental time (Table 1). Clearly N-CAM is one of the earliest markers for differentiation following gastrulation, and it may play a role in early inductive interactions as well as in shaping of early organ rudiments. This is not inconsistent with the later deployment of the molecule for specific neuron-neuron and neuromuscular interactions. Indeed, only a few different CAM's may have been evolved for such early and critical embryonic inductive interactions, to be modified later by local surface modulation for formation of detailed histogenetic patterns in a variety of organs. A reasonable prediction based on this idea is that L-CAM will be found on many endodermal anlagen early in embryogenesis. Indeed, it would not be surprising if, in addition, L-CAM and uvomorulin (*16*) were shown to be closely related or identical.

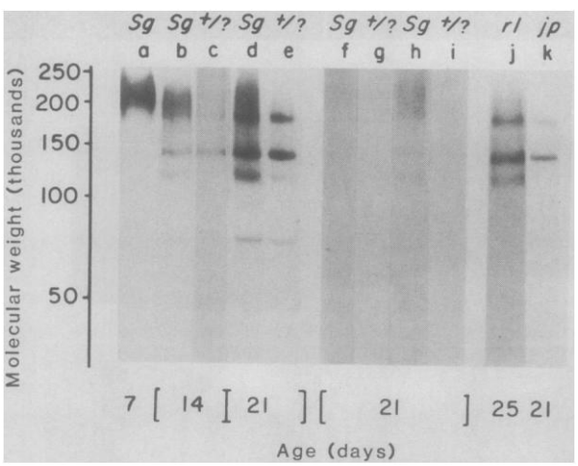

Fig. 7. Conversion of N-CAM from embryonic (E) to adult (A) forms in *staggerer* mice (*sg*) and other mouse neurological mutants. Immunoprecipitate of cerebellar membrane extracts were subjected to polyacrylamide gel electrophoresis in sodium dodecyl sulfate. The same amounts of membrane vesicle protein and the same amounts of antibodies in antibody excess were used in all immunoprecipitations. (Lanes a to e) *staggerer* (*sg*) and littermates (+/?—a mixture of wild type +/+, and heterozygote +/*sg* animals) at various postnatal times. Rabbit antibody to mouse N-CAM was used. (Lanes f and g) A monoclonal antibody (15G8) reactive with sialic acid in the E form but nonreactive with the A forms was used. (Lanes h and i) A monoclonal antibody (9E11) to the protein portion of N-CAM was used. This antibody was reactive with N-CAM from embryonic cerebellum but not with N-CAM from adult cerebellum. (Lane j) *reeler* (*rl*) cerebellum with rabbit antibody to N-CAM: (lane k) *jimpy* (*jp*) cerebellum with rabbit antibody to N-CAM. (Lanes b and d) Failure of complete N-CAM conversion in *staggerer* at 14 and 21 days to the three normal A forms with molecular weights of 180,000, 140,000, and 120,000 (as shown, for example, in lane e). Two different antigenic determinants characteristic of the E form from cerebellum (lanes f and h) were retained in *staggerer*.

Conclusions

While the study of cell-cell adhesion molecules is still at its beginning, a number of facts and principles have emerged that may be stated succinctly here for future revision and test:

Cell-cell adhesion, cell-substrate adhesion, and intercellular junctions appear to be mediated by completely different families of molecules; of these systems, CAM's appear to be the most cell-specific. There are at least three epochs in specific CAM function: the early embryonic related to inductive events and formation of organ rudiments, the embryonic related to detailed histogenesis, and the adult, which may be concerned with surface regulation (*2*) of cellular metabolic states.

N-CAM appears to be the major neuron-neuron and neuron-muscle adhesion molecule in many vertebrate species and is the best studied of the CAM's to date. This molecule has been found in human brain, but its role in human disease has not yet been explored. During development, N-CAM undergoes local cell surface modulation, the E → A conversion. So far, the most striking support for modulation theories of cell adhesion comes from the observation that the normal conversion of N-CAM to various A forms fails to occur in the cerebellum of homozygous *staggerer* mice.

It is premature to consider in detail whether exquisite neural mappings, seen for example in the retinotectal projection, can be accounted for by modulation of N-CAM alone. But it is relevant that certain recent analyses (*40*) discount strict chemoaffinity theories and are consistent with local cell surface modulation (*2*). Reformulation and experimental tests of such models in terms of CAM action are now feasible. Whatever the

specific outcome, the present evidence on CAM's already suggests that modulation theories more correctly describe the bases in adhesion of cell-cell patterning than do strict chemoaffinity theories. This conclusion is in accord with, and central to, the assumptions (*41*) of neuronal selection theories of brain function.

A number of other CAM's are being studied, for example, L-CAM and GN-CAM. The relation of L-CAM function to gene expression and control of protein synthesis in the liver may shed new light on surface modulation (*2, 22*). The analysis of GN-CAM and N-CAM and their manipulation in vivo may play a significant part in attempts to obtain neural regeneration in the adult central nervous system. Above all, successful correlation of the detailed chemical structures of various CAM's with their functions in different developing organ systems should mark an important step toward the realization of a soundly based molecular embryology.

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