Neural Cell Adhesion Molecule Mediates Initial Interactions Between Spinal Cord Neurons and Muscle Cells in Culture

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ABSTRACT Previous studies in this laboratory have described a cell surface glycoprotein, called neural cell adhesion molecule or N-CAM, that appears to be a ligand in the adhesion between neural membranes. N-CAM antigenic determinants were also shown to be present on embryonic muscle and an N-CAM-dependent adhesion was demonstrated between retinal cell membranes and muscle cells in short-term assays. The present studies indicate that these antigenic determinants are associated with the N-CAM polypeptide, and that rapid adhesion mediated by this molecule occurs between spinal cord membranes and muscle cells. Detailed examination of the effects of anti-(N-CAM) Fab' fragments in cultures of spinal cord with skeletal muscle showed that the Fab' fragments specifically block adhesion of spinal cord neurites and cells to myotubes. The Fab' did not affect binding of neurites to fibroblasts and collagen substrate, and did not alter myotube morphology. These results indicate that N-CAM adhesion is essential for the in vitro establishment of physical associations between nerve and muscle, and suggest that binding involving N-CAM may be an important early step in synaptogenesis.

The interaction between nerve and muscle in culture has been extensively studied as a model system for synaptogenesis (1-3). This work has led to the identification of factors (4, 5) that influence the growth of nerves to muscles, and to a better understanding of the components and structure of the neuromuscular synapse (2-5). Relatively little is known, however, about the substances or mechanisms involved in the initial physical interaction between nerve and muscle. Factors that promote growth of nerves to muscle, such as cell-conditioned media (6) or extracellular matrix components (4), might be involved in the binding of nerve to muscle. In addition, the acetylcholine receptor itself, which is known to exist in aggregates on the muscle before synaptogenesis (1, 3), might influence nerve-muscle adhesion. As yet, however, there is no evidence either that the trophic factors are associated with cell adhesion, or that pre-existing acetylcholine receptor clusters influence the location of synapses (4).

Studies in culture suggest that adhesive interactions and transient synapses to muscle can be formed using nerve cells from a variety of tissues including parasympathetic (7) and sympathetic ganglia (8), cerebral cortex (9), and clonal neuroblastoma x glioma hybrid cells (10-12). Furthermore, the neuroblastoma x glioma hybrid, which is derived from mouse, not only binds to muscle from mouse but also to muscle from rat and chick (13). These findings imply that the mechanism of initial binding between neurons and muscle does not possess a stringent tissue or species specificity. Rather, it is likely that a more general adhesive interaction between nerve and muscle establishes an initial physical contact, and that synaptic specificity is determined by, or in combination with, other events.

These properties of initial nerve-muscle interactions raised the possibility that they involve the cell surface protein called neural cell adhesion molecule or N-CAM (14-16). The specific affinity of N-CAM for nerve and muscle cells suggests that it is a ligand in the formation of cell-cell bonds (17) and studies using antibodies to N-CAM have demonstrated that the function of this molecule is required for nerve cell sorting (18, 19), and neurite fasciculation (20, 21). N-CAM is present in a variety of species (22, 23) and appears early during embryogenesis in a variety of sites including those that have been determined to differentiate into striated muscle (24). Moreover, antigenic determinants recognized by antibodies against N-CAM have also been shown to be present on the surfaces of embryonic muscle cells, and Fab' fragments prepared from these antibodies inhibit binding of retinal cells, brain membrane vesicles, or reconstituted vesicles containing N-CAM to such muscle cells in short-term assays (25). The purpose of these studies was to identify and characterize further the N-CAM antigens on muscle cells, and to evaluate...
the role of N-CAM in the interactions of spinal cord neurons with skeletal muscle in culture.

MATERIALS AND METHODS

Preparation of Cells and Tissue: Muscle cells were obtained from breast muscle of 12-d chicken embryos by trypsinization (0.1% trypsin [Gibco Laboratories, Grand Island, NY], 0.02% EDTA, and 20 μg/ml DNase I [ Worthington Biochemical Corp., Freehold, NJ] in calcium and magnesium-free medium [CMF], 30 min, 37°C), followed by centrifugation in medium (Eagle's Minimal Essential medium with 10% horse serum; both from Gibco Laboratories) with a Pasteur pipette. Undissociated tissue was removed by low-speed centrifugation and the cells were pelleted through 3.5% bovine serum albumin (BSA, fraction V, Sigma) and concentrated into saline (PBS), pH 7.4. The isolation of the immunoglobulin by ion-exchange chromatography and its cleavage by pepsin to produce monovalent Fab' fragments has been described previously (26). Monoclonal antibody to N-CAM (No. 1) was prepared as reported by Hoffman et al. (16). Immunoprecipitation of 125I-labeled nerve membrane proteins by the rabbit antibodies and mouse monoclonal antibodies both yielded material that migrated in SDS-PAGE as the single diffuse band previously identified as N-CAM (16).

Cultures and Cinematography: In most experiments, muscle cells were plated in medium supplemented with 1/20 vol of chick embryo extract (Gibco Laboratories). The collagen was prepared by extraction of rat tail tendons with 0.1% acetic acid for 48 h at 4°C. Spinal cords were dissected from 12-d chick embryos and cut into 0.5-mm fragments. Cells from spinal cords were prepared by trypsinization and centrifugation of the minced tissue, as described above for muscle cells except that the preplating step was omitted.

Fibroblasts were obtained by trypsinization and centrifugation of skin from 12-d chick embryos as described above and grown in Dulbecco's modified Eagle's medium (Gibco Laboratories) containing 10% fetal calf serum (Gibco Laboratories).

Antibodies: Rabbit antibodies to affinity-purified N-CAM (16) were obtained after 5-10 monthly intersperimertal and intramuscular injections each of 50 μg of antigen in Complete Freund's Adjuvant (first injection), Incomplete Freund's Adjuvant (second through fourth injection) or phosphate-buffered saline (PBS), pH 7.4. The isolation of the immunoglobulin by ion-exchange chromatography and its cleavage by pepsin to produce monovalent Fab' fragments has been described previously (26). Monoclonal antibody to N-CAM (No. 1) was prepared as reported by Hoffman et al. (16). Immunoprecipitation of 125I-labeled nerve membrane proteins by the rabbit antibodies and mouse monoclonal antibodies both yielded material that migrated in SDS-PAGE as the single diffuse band previously identified as N-CAM (16).

RESULTS

Detection of N-CAM on Muscle and Spinal Cord Cells

Previous studies using immunofluorescence techniques demonstrated that N-CAM antigenic determinants are present in all nerve tissues, including spinal cord (20, 24), as well as on the surface of cultured myoblasts and myotubes from 12-d chick embryos (25). The staining of myotubes was uniform and significantly less bright than that of nerve cells. To establish the chemical identity of the molecules with which these determinants were associated, immunoprecipitation with anti-(N-CAM) monoclonal antibodies followed by PAGE in SDS was carried out (Fig. 1a). The material immunoprecipitated from a detergent extract of spinal cord migrated as a diffuse band of low mobility, closely resembling the N-CAM previously identified in brain tissue (16). The material immunoprecipitated from muscle, on the other hand, had a higher average mobility and in this respect resembled the form of N-CAM found in retinal tissue (16). In a similar experiment, the immunoprecipitates were treated with neuraminidase and then analyzed by PAGE in SDS (Fig. 1b). With this treatment, N-CAM from brain and spinal cord were indistinguishable; both contained bands of 170,000 and 140,000 daltons. Muscle N-CAM migrated in these gels as a doublet centered around 140,000 daltons (Fig. 1b). In preliminary experiments, radioliodination of myotubes in culture followed by immunoprecipitation with anti-(N-CAM) antibodies showed results similar to those indicated above for muscle tissue.

Rapid Binding of Spinal Cord Membrane Vesicles to Muscle Cells

An earlier investigation of N-CAM-mediated adhesion in nerve-muscle interactions (25) allowed measurement of the rate of binding and evaluation of the specificity without ambiguity, but for technical reasons was confined to the use of retinal and brain tissue. To demonstrate that the same binding occurs in the more physiologically relevant spinal cord-myotube combination, we examined the effect of anti-(N-CAM) on adhesion of fluorescent spinal cord membrane vesicles to monolayers of cultured myotubes in a short-term assay involving a 30-min incubation with constant mixing to avoid nonspecific adhesion (Fig. 2). In the absence of anti-(N-CAM) Fab', the surface of the myotubes became covered with the fluorescein-labeled spinal cord vesicles, but no bind-
ing was observed between the vesicles and the fibroblasts (Fig.
2, a and b). These vesicles were firmly bound to the myotubes
and could not be removed by washing of the monolayer. The
adhesion of vesicles to the myotubes appeared to be specific
in that no binding occurred in the presence of affinity-purified
anti-(N-CAM) Fab' (20 µg/ml; Fig. 2, c and d). Cells with a
fibroblastlike morphology (Fig. 2, a and b), however, did not
bind spinal cord vesicles. This result is consistent with pre-
vious studies that showed that brain vesicles bound to cells
that had N-CAM on their surface but not to fibroblasts that
have no detectable N-CAM (20, 25).

Effect of Anti-(N-CAM) on Neurite Outgrowth
from Spinal Cord Explants in the Presence
of Myotubes

These experiments were carried out by culturing a small
piece of spinal cord tissue on an established myotube mono-
layer; anti-(N-CAM) antibodies had little or no effect on
muscle cell aggregation or on the formation or appearance of
the myotube monolayer (unpublished data). The extent and
pattern of neurite outgrowth from the spinal cord explants on
a sparse myotube monolayer in the presence of anti-(N-CAM)
Fab' or Fab' from unimmunized rabbits is illustrated in Figs.
3 and 4. These results are best described in contrast with the
outgrowth from spinal cord explants alone. During culture
on collagen-coated substrates, a halo of flat cells (probably
glial cells) develops around the explant and the extent of
neurite outgrowth is predominantly limited to this region in
the presence and the absence of anti-(N-CAM) (Fig. 5, b and
d).

The nerve-muscle cultures with nonimmune Fab' (Figs. 3,
a and c; 4a) displayed a consistent morphology in which
neurites radiated far beyond the flat cell halo and the pattern
of nerve outgrowth appeared to be determined by the arrange-
ment and shape of the myotubes. Nearly all the neurite tips
were associated with muscle cells, and myotubes located near
the explant were often covered with and connected by a
network of nerve processes. Time-lapse cinematography was

![Figure 1](image1.png)

**Figure 1** PAGE in SDS of 125I-labeled cell surface components
recognized by monoclonal antibodies against N-CAM. Lactoper-
oxidase-radioiodinated N-CAM was isolated from the indicated
sources as described in Materials and Methods, electrophoresed
on polyacrylamide gels, and the samples were visualized by auto-
radiography. (a) Samples are from brain (B), spinal cord (SC), retina
(R), and breast muscle (M). (b) Samples were similar to (a), but were
treated with 10 µl of neuraminidase (Calbiochem-Behring Corp., 1
IU/ml) for 8 h at 37°C (39) before boiling. Molecular weight
standards were from Bio-Rad Laboratories (Richmond, CA) and
their migration positions in the gel are indicated.

![Figure 2](image2.png)

**Figure 2** Binding of fluorescent membrane vesicles obtained from spinal cord
to cultured myotubes. (a and c) Phase-
contrast microscopy; (b and d) fluores-
cence microscopy. Binding occurred
during a 30-min incubation at 37°C. In
the presence of 20 µg/ml of nonimmune
Fab' (a and b) vesicle binding was ob-
served to myotubes but not to cells with
a fibroblastlike morphology (f), which
are below the focal plane in this micro-
graph. (c and d) Preincubation of the
vesicles and the myotube monolayers
with 20 µg/ml of affinity-purified anti-
(N-CAM) Fab' inhibited vesicle binding.
× 234.
used to monitor the dynamic behavior of spinal cord explants cultured on myotube monolayers. This analysis revealed that the observed network of neurites resulted from the following series of events.

When a neurite initially emerged from spinal cord tissue, it either grew rapidly along a myotube lying under or alongside the explant or, in the absence of a myotube, probed the surrounding collagen-coated substrate. In the latter case, the rate of growth was slow, with frequent slipping and even retraction, as previously noted in cultures of spinal ganglia (21). If, however, sufficient extension occurred to allow contact with a myotube, more rapid growth ensued along the surface of the muscle cell. Once a neurite connection between the explant and a myotube was established, it often served as a bridge for rapid outgrowth of many other neurites, thus forming a fascicle. Subsequently, the growth cones of the individual fibers migrated independently along the surface of the myotube, causing the fascicle to split apart and form a network of fibers as shown in Fig. 3, a and c. In the course of these events, growth cones would also occasionally depart from a myotube and in a similar manner form bridges and neurite networks between myotubes. As a specificity control for the anti-(N-CAM) effects described below, cultures were incubated in the presence of polyspecific antibodies raised against fibroblasts that also cross-react with neural and muscle cells, or with anti-(N-CAM) that had been neutralized with purified N-CAM (16). These cultures were indistinguishable from those containing nonimmune Fab', except that the polyspecific antibody caused a slight rounding of the fibroblastlike cells on the substrate.

The presence of anti-(N-CAM) Fab' in the medium produced dramatic and varied changes in the appearance of these cultures, the most consistent being a decrease in overall extent of neurite outgrowth, and a reduction in the number of nerve-nerve and nerve-muscle contacts. In most cases, the neurites grew out only a short distance from the explant (often corresponding with the extent of flat-cell migration from the spinal cord explant) and formed only a few transient connections with a myotube (Fig. 3, b and d). Our previous observations with spinal ganglia (18, 21) indicated that the rate of outgrowth on the collagen substrate and the motility of growth cone filopodia are not diminished by anti-(N-CAM) Fab'. In fact, the analysis of time-lapse cinematographic records of cultures displaying neurite outgrowth on collagen indicated that the rate of neurite bundle elongation was slightly increased by anti-(N-CAM) Fab', from 0.07 ± 0.02 mm/h to 0.09 ± 0.02 mm/h (measured during the 15–20-h period of culture). The small increase in the rate of neurite elongation in the presence of anti-(N-CAM) may be attributed to a decrease in neurite fasciculation, which allows thinner neurites to develop with more tension and elongate somewhat more rapidly as suggested previously for dorsal root ganglion.
FIGURE 4 Nerve-muscle cultures with a more adhesive substrate. (a) Control culture with 200 μg of collagen substrate per dish and with medium containing Fab' from unimmunized rabbits. (b) Culture on a substrate containing 200 μg of collagen per dish with medium containing anti-(N-CAM) Fab'. (c) Culture with anti-(N-CAM) Fab' in which a large region of the collagen substrate has been covered by flat cells from the explant. Note that extensive outgrowth occurs in each case, but that in the presence of anti-(N-CAM) Fab' the path of neurites growing on the 200 μg/dish collagen substrate (b) was seldom altered by the myotubes (arrows), and that in cultures with flat cells (c) the neurites adhered preferentially to the flat cells rather than the muscle cells. As a result, the network of fibers interconnecting the myotubes in a are not observed in b and c. M, breast muscle; SC, spinal cord; FC, flat cell. × 82.

cultures (21). In the case of the spinal cord, however, the extent of outgrowth appears to have been limited because in the presence of anti-(N-CAM) Fab' the neurites were unable to use the myotubes as a stable anchor from which further growth could occur.

Some of the variations in morphology of the cultures with anti-(N-CAM) Fab' (Fig. 4, b and c) appeared to reflect heterogeneity in the adhesivity of the substrate. When the amount of collagen on the culture dish was increased from 50 to 200 μg, substantial neurite outgrowth was observed even in the presence of anti-(N-CAM). However, as revealed by cinematography, the myotubes were virtually ignored, with the growth cones often growing over or under the myotubes without changing direction (the arrows in Fig. 4b indicate examples of neurites crossing over or under myotubes). A different example of increased adhesivity occurred when in some cultures, a broad region of flat cells developed around the explant. In such cultures, the neurites grew out in the presence of anti-(N-CAM) Fab' and formed a meshwork over the surface of the flat cells, but failed to extend significantly beyond them to adjacent myotubes (Fig. 4c). In both of these cases, the intricate network of neurites seen on or near myotubes in the presence of nonimmune Fab' (Fig. 4a) was not observed.

These results suggested that the effects of anti-(N-CAM) Fab' were caused by a specific inhibition of nerve-muscle interaction. Because anti-(N-CAM) Fab' also inhibits the bundling of nerve fibers, it was necessary to evaluate the contribution of nerve-nerve interactions. Therefore, observations were made on cultures of spinal cord explants without myotubes. When the myotubes were replaced by a sparse monolayer of fibroblasts, bundles of neurites were observed that had extended beyond the halo of flat cells to fibroblasts (Fig. 5a). The anti-(N-CAM) Fab' primarily caused a decrease in neurite fasciculation (Fig. 5c) as previously observed (18, 21), but did not reduce the extent of outgrowth or prevent the adhesion of neurites to the fibroblasts. Nerve-fibroblast adhesion also differed from nerve-nerve and nerve-muscle adhesion in that it occurred primarily at the tip of neurites. This property was revealed by the observation that perturbation of the medium with a pipette caused the entire neurite to oscillate between its point of emergence from the explant and its distal attachment to a fibroblast. In cultures of spinal cord explants alone on a collagen substrate the only noticeable effect of anti-(N-CAM) Fab' was a reduction in the average size of nerve bundles. Together these results suggested that the effects of anti-(N-CAM) Fab' on cultures with myotubes were not caused by inhibition of neurite-neurite adhesion alone, or by direct perturbation of nerve-fibroblast or nerve-substrate interactions.

Cultures with Cells Obtained from Spinal Cord

The interaction of nerve and muscle was also observed in cultures of individual spinal cord cells with myotubes. These studies had the advantage that at low nerve cell densities, relatively little fasciculation occurred among neurites, thus mitigating the contribution of nerve-nerve interactions to our observations. The results of these experiments are presented both as representative photomicrographs (Fig. 6) and as a statistical tabulation (Table I). In the presence of nonimmune Fab', nearly all the nerve cells adhered to and flattened out along the muscle cell surface (Fig. 6a), and most of the nerve processes grew along the length of the myotubes (Fig. 6b). In contrast, with anti-(N-CAM) Fab' the majority of nerve cell bodies became attached to the substrate (Fig. 6c). The number of visible nerve fibers, that is those extending between myotubes, nerve cells, or the substrate, was about three times greater than in the control cultures (Table I).

As in the studies with nerve explants, experiments without...
myotubes were also used as a control for specificity (Fig. 6, d and e). Anti-(N-CAM) had no detectable effect on the appearance of these cultures, as previously reported for cells from spinal ganglia (21), suggesting that the antibody does not directly interfere with the elongation of neurites along the substrate.

DISCUSSION

The results of these studies confirm that the N-CAM glycoprotein is present on chick embryo myotubes and spinal cord neurons, and that it mediates specific binding between these two cell types. They also suggest that the initial adhesive interaction between nerve and muscle in culture requires the function of this molecule.

The conclusion that an N-CAM-specific mechanism exists for nerve-muscle adhesion is primarily based on the rapid binding to myotubes of retinal cells (25) or membrane vesicles from spinal cord, and the inhibition of this binding by anti-(N-CAM) Fab'. While spinal cord membranes represent a more physiologically relevant choice than retinal cells, it should be noted that, in vivo, only a small percentage of spinal cord neurons actually innervate muscle. Therefore our studies more accurately reflect a description of neuron-myotube interactions in general rather than of motor neurons with specific muscles.

In previous studies on the binding activity of the purified N-CAM glycoprotein (17), it was noted that adhesion involving N-CAM was detected between two different cells only if both had this molecule on their surfaces. This and other observations led to the working hypothesis that two N-CAM molecules are required to form each bond between the cells. The identification of the N-CAM polypeptide on muscle cells, and the demonstration of an N-CAM-specific adhesion between nerve and muscle, provide additional support for this hypothesis. N-CAM does not appear to play a major role in the aggregation of muscle cells since, in preliminary experiments, we detected only a minor effect of anti-(N-CAM) Fab' on muscle cell aggregation (10–20% inhibition). This could result from the apparent lower density of N-CAM on muscle, possible preferences in binding among different forms of N-CAM, or the domination of muscle-muscle interactions by another adhesion mechanism, perhaps one associated with the fusion process.

Adhesive interactions between embryonic spinal cord and skeletal muscle dominate the morphology of the neurite outgrowth in culture; specific inhibition of N-CAM function by Fab' effectively prevented this mode of nerve-muscle recognition. While it has not been established that N-CAM-mediated adhesion is required for synapse formation in developing tissue, it is likely that physical adherence represents an
early step in this process. It has been observed that nerve-muscle contact as well as a variety of substances can induce clustering of receptors for acetylcholine (29–33), and we are examining the possibility that N-CAM may be involved in this phenomenon. Preliminary studies suggest, however, that N-CAM is not preferentially located in the receptor clusters.

The cinematographic experiments emphasized the dynamic interplay between nerve-nerve, nerve-muscle, and nerve-substrate interactions. Recent studies on the anatomical mapping of "pioneer" nerves in insects (34, 35) have led to the hypothesis that specific nerve guidance could result from an ordered array of such "stepping stones." In this process, growth cones actively probe their environment in search of an appropriate adhesive target; once found, the elongating neurite anchors to the target, and these events can then repeat themselves. Using nerve-muscle cultures as a model, we have directly observed phenomena that resemble such a process. For example, nerves from the spinal cord repeatedly extended into various regions of surrounding substrate until one found a myotube; after a period of time, during which translocation of the growth cone along the myotube occurred, this nerve again explored the surrounding substrate until another myotube was found. As each such bridge was established, many other nerves followed this initial route, thereby forming a nerve "tract" between the explant and a particular area of the culture dish. Inasmuch as N-CAM function is required for both nerve-muscle and nerve-nerve adhesion, its inhibition by anti-(N-CAM) Fab' prevented the establishment of such pathways as well as the recruitment of other neurites into bundles.

The difference in electrophoretic mobility of the N-CAMs from 12-d embryonic muscle and 7-d spinal cord nerve suggests that the molecules isolated from these tissues have somewhat different physical properties. A similar heterogeneity has been observed in comparing embryonic retina with brain, and appears to result from variation in the sialic acid content of the glycoprotein (16). Muscle N-CAM contains sialic acid since treatment with neuraminidase produced sharper bands of higher mobility on SDS gels. N-CAM isolated from adult brain contains less sialic acid than that isolated from embryonic brain (36), suggesting that this variation may reflect both tissue specificity and developmental changes in the molecule (37). A failure in the conversion of

| Fab'   | Total cells scored | Bound to myotube | Not in contact with myotube | Number of visible processes |
|--------|--------------------|------------------|-----------------------------|-----------------------------|
| Nonimmune | 908                | 87 ± 6           | 13 ± 2                      | 288                         |
| Anti-(N-CAM) | 984                | 39 ± 3           | 61 ± 4                      | 1,008                       |

* Ten areas representing different locations in duplicate cultures were scored.
* The standard deviations observed in ten areas are indicated.
* The size of nerve bundles was similar in both types of culture (see Fig. 6), and each neurite or bundle was scored as one process.

Table I. (d) and (e) represent cultures of spinal cord cells on collagen alone in the presence of nonimmune and anti-(N-CAM) Fab', respectively. No morphological differences between these cultures were detected. M, breast muscle. a, x 82; b and c, x 328; d and e, x 82.
this molecule from the embryonic to the adult state has been correlated with connectional defects in the cerebellum of staggerer mice (38), and it will be interesting to look for comparable disorders in nerve-muscle interaction.

As noted previously (25), the detection of N-CAM on cells of mesodermal as well as ectodermal origin suggests that its expression reflects morphogenetic requirements rather than specific cell lineage from a particular germ layer. This hypothesis is also suggested by the observation that, in the early embryo, N-CAM is present not only in structures destined to form the nervous system and mesoderm, but also has a transient appearance in placodes and in precursors of the mesonephric tubule (24). While the developmental function of N-CAM in these tissues is as yet unknown, they are thought to be sites of intense inductive interactions. It will be of considerable interest, therefore, to evaluate the importance of N-CAM both in inductive events between nerve and muscle and in synaptogenesis.

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