Rapid Adhesion of Nerve Cells to Muscle Fibers from Adult Rats Is Mediated by a Sialic Acid–binding Receptor

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Abstract. Single viable muscle fibers isolated from adult rats by collagenase digestion rapidly bind dissociated spinal neurons or PC-12 cells but not a variety of other cells tested. The adhesion process is calcium-independent, temperature-sensitive, and is not blocked by pretreating cells with inhibitors of energy metabolism or actin polymerization. Adhesion is mediated by a carbohydrate-binding protein and can be inhibited by *N*-acetylneuraminic acid or mucin, a glycoprotein with high sialic acids content. The hapten inhibitors do not dissociate cells if added after aggregation has occurred. Experiments to block adhesion by pretreatment of cells with either neuraminidase or mucin show that the sialic acids–rich moiety is on the nerve cells, while its receptor is on the muscle fibers.

THE first contact between nerve and muscle is the attachment of the nerve terminal to the surface of the muscle fiber. This initiates a series of interactions during which the two cells become mutually dependent upon each other for their survival. Surface molecules mediating this attachment may serve as triggers for subsequent events and are thus central to our understanding of neuromuscular interaction.

Several types of cell surface molecules have been implicated in neuronal adhesion. Neural cell adhesion molecule (N-CAM)[1] is a membrane glycoprotein widely distributed throughout the nervous system and involved in calcium-independent aggregation between neural cells (19, 43). N-CAM is also present on skeletal myoblasts and myotubes, and univalent antibody to the molecule blocks the binding of spinal cord cells or vesicles containing N-CAM to cultured myotubes (22, 44), suggesting that similar mechanisms are involved in nerve–nerve and nerve–muscle adhesion. Another membrane glycoprotein named r-cognin has been found to promote adhesion between neural retina cells (24, 25). R-cognin is tissue-specific, but similar aggregation-enhancing factors can be isolated from other tissues including spinal cord and skeletal muscle (26). These latter activities have not yet been characterized. Glycolipids are a major component of the neuronal cell surface and have been found to undergo developmental changes during neurogenesis (34). A particular ganglioside, 18B8, undergoes quantitative and qualitative changes in the synaptic layer of the retina during its maturation (23). Quantitative differences in ganglioside content have also been found between cholinergic and noncholinergic synaptic membranes (33). There is some evidence that glycolipids are involved in the development of the neuromuscular junction since addition of GM_1 or globoside to the medium of co-cultures of muscle and spinal cord results in suppression of spontaneous end plate potentials in the myotubes (40).

In the present study we have developed an experimental approach to the analysis of nerve–muscle adhesion. When muscle fibers from adult rats are mixed with PC-12 cells or spinal neurons, the two cell types rapidly form co-aggregates that can be quantitatively measured by prelabeling the nerve cells. Pure populations of both cell types can be obtained, and the adhesion assay involving separation of the co-aggregates from unbound cells is rapid and efficient. Results show that adhesion is mediated by a receptor on the surface of the muscle fibers that binds to a sialic acids–containing ligand on the nerve cells. Adhesion can be eliminated by removal of the basal lamina from the muscle fiber.

Materials and Methods

Materials

Alpha-bungarotoxin was purified from snake venom (Sigma Chemical Co., St. Louis, MO) by chromatography on Sephadex G-50 (Pharmacia Fine Chemicals, Piscataway, NJ) followed by carboxymethyl Sephadex as described by Berg et al. (4) and labeled with fluorescein isothiocyanate (18). Biological activity of the purified toxin was tested with a rat phrenic nerve–diaphragm preparation. Purified neuraminidase (Sigma type V) had no detectable protease activity with Azocoll (Calbiochem-Behring Corp., La Jolla, CA) at 50 times the concentration used in this study. Pig gastric mucin (Sigma Chemical Co., 0.75–1% sialic acids content) was purified by elution from a column of Sephacryl S-300 (36). Sialic acids were estimated by the periodate–resorcinol method of Jordian et al. (29) and protein was determined using the method of Lowry et al. (35). Pronase was from Calbiochem-Behring Corp.; all other chemicals were from Sigma Chemical Co.

Cells

Muscle fibers were prepared from the toe muscle (flexor digitorum brevis) of adult rats as described previously (3, 10). Briefly, the muscles were stripped of epimysium and treated for 2 h at 37°C in Earle's balanced salt solution

1. *Abbreviations used in this paper:* NANA, *N*-acetylneuraminic acid; N-CAM, neural cell adhesion molecule.

containing a mixture of 0.6 mg/ml collagenase and 0.4 mg/ml neutral protease both purified from crude collagenase (27). After digestion, the tendons were separated, and individual fibers were liberated by gentle trituration with a wide-mouth pipet. The fibers were separated from non-muscle cells by repeated 1 *g* sedimentation through a column of medium, and an aliquot was counted by microscopy. The muscles from one rat yield ~ 3×10^4 fibers. The fibers retain normal morphology, are covered with a basal lamina, and remain viable for at least several weeks in culture (9, 10, 11). The fibers were dissociated and purified in Dulbecco's phosphate-buffered saline (PBS) with 10% horse serum and cultured in Eagle's minimum essential medium with 10% horse serum and 1% antibiotic–antimycotic mixture. All culture reagents were from Gibco, Grand Island, NY.

Nervous tissue was obtained from 14–19-d rat embryos or 8-d chick embryos. After removal of the meninges, the tissue was minced into 1-mm^3 pieces and treated with 0.1% trypsin in Ca^{++}-Mg^{++}-free Earle's balanced salt solution for 1.5 h at 37°C. Spinal ganglia were treated without mincing. Cells were dissociated by trituration, washed, and grown in minimum essential medium with 10% horse serum and 5% fetal calf serum plus 1% antibiotics.

Rat pheochromocytoma cells (PC-12) were obtained from Dr. L. Greene (21) and were grown in RPMI 1640 with 15% fetal calf serum, 5% horse serum, and 1% antibiotics. PC-12 cells were passaged by trituration and were not exposed to trypsin. More than 99% of the cells were viable as measured by dye exclusion (0.05% nigrosin in PBS). Since these cells tend to grow in clumps, special care was taken to obtain monodisperse cells for use in quantitative adhesion assays. Before use, the suspended cells were filtered through two 10-μm nylon filters (Tetco, Inc., New Britain, CT) to remove clumped cells that were not dissociated during the trituration. Greater than 80% of the cells in these preparations were monodisperse with most of the remainder as pairs. A crude membrane fraction was prepared from PC-12 cells as described by Ratner et al. (42). Cells were broken by Dounce homogenization (B pestle) in buffer (10 mM Tris, pH 7.4, 250 mM sucrose, 0.1 mM $MgCl_2$, 17 mU/ml aprotinin) and centrifuged at 2,000 *g* for 10 min. The supernatant was centrifuged at 32,000 *g* for 90 min in a SW39 rotor (Beckman Instruments, Inc., Palo Alto, CA). The membrane pellet was resuspended in PBS by homogenization, and the non-membrane supernatant was dialyzed against PBS. These two fractions were used in adhesion assays.

Most of the experiments reported here were done using PC-12 cells because of purity and ease of preparation. Each type of experiment, however, was also done with spinal cord neurons with similar results.

Two lines of human neuroblastoma cells (NGP and NMB) from Dr. M. Goldstein (13) and HeLa cells obtained from Dr. L. Tolmach were grown in minimum essential medium with 20% fetal calf serum and 1% antibiotics. Purified rat embryo Schwann cells obtained from Dr. R. Bunge (50) were grown as described for the spinal cord cells. Other rat embryo cells were prepared by trypsin disaggregation of the appropriate organs or tissues. Freshly obtained erythrocytes were treated with crystalline trypsin and glutaraldehyde as described by Nowak et al. (39) before use in binding assays.

Adhesion Assay

Single muscle fibers were used in adhesion assays just after dissociation, but cells exposed to trypsin were allowed to recover for 24 h. For recovery the cells were plated at low density on soft gels of rat tail collagen (12) diluted to 0.5–1 mg/ml and gelled at 37°C after adjusting the pH and salt concentration (20). For use in adhesion assays the cells were removed from the collagen by gentle pipetting. Muscle fibers and test cells were combined in 24-well tissue culture plates (No. 3047, Falcon Labware, Oxnard, CA) in 0.5 ml PBS with 10% horse serum. Unless otherwise noted, each well contained ~10^3 muscle fibers and ~10^5 nerve cells. The plates were placed on a platform rotating at 4 rpm and tilted 20°. This was designed to gently mix the cells at the bottom of the wells. More rapid agitation suspends the nerve cells but not the muscle fibers and results in lower efficiency of adhesion. The cells were mixed for 10 min at room temperature (21–23°C) unless otherwise noted and scored visually with an inverted phase-contrast microscope for semiquantitative estimation of adhesion.

For quantitative measurements, test cells were first labeled by overnight exposure to medium containing [^{3}H]L-leucine (3 μCi/ml, 5 Ci/mmol, New England Nuclear, Boston, MA). After labeling, cells were washed and manipulated in chase medium containing 1 mM L-leucine. Cells were counted with a hemocytometer. After allowing adhesion between triplicate aliquots of cells and muscle fibers as above, the co-aggregates were separated from unbound labeled cells by filtration. The contents of each culture well were transferred to a filter well assembly consisting of a 12-mm disk of 46-μm mesh monofilament nylon screen (Small Parts Inc., Miami, FL) held by a sleeve at the end of a section of glass tubing (12-mm diam × 20-mm long). The sleeve was made from a culture tube cap (No. 2054, Falcon Labware) with the end punched out leaving a 1-mm shoulder. The filter wells were mounted in a carrier (Fig. 1), and the co-aggregates were washed with 10 dips in each of two changes of PBS with 10% horse serum and one change of PBS alone. Each filter was then removed and transferred to a scintillation vial for determination of radioactivity. The mesh size was selected on the basis of experiments to determine labeled co-aggregates retained vs filter porosity. A 46-μm pore filter retains all muscle fibers but passes unbound test cells. In each experiment duplicate filter wells contained test cells alone to control for possible self-aggregation in the absence of muscle fibers. In all cases, these control filters contained <0.5% of the radioactivity of the wells with muscle fibers added. For counting, the filters were placed in 0.5 N NaOH overnight, neutralized, and counted in 3a70 (Research Products International Corp., Elk Grove Village, IL) using a Beckman scintillation counter.

Treatment of Cells With Mucin or Neuraminidase

Muscle or nerve cells were exposed to 1 mg/ml pig gastric mucin in PBS for 15 min at room temperature. Neuraminidase was used at 0.2 mg/ml Hank's balanced salt solution brought to pH 5.5 by gassing with CO_2. Digestion was done for 1 h at 37°C. After both treatments cells were rapidly washed three times with PBS containing 10% horse serum. As a control for the low pH used during digestion, cells were incubated under the same conditions but with enzyme inactivated by boiling.

Microscopy

Fluorescent microscopy was done using a Zeiss microscope with an epifluorescence condenser and halogen lamp. Specimens were mounted in Aqua-mount (Lerner Laboratories, New Haven, CT). For scanning electron microscopy, co-aggregates of nerve cells and muscle fibers were centrifuged (150 *g*) onto polylysine-coated coverslips, fixed in 3% glutaraldehyde in PBS, dehydrated in methanol, critical point dried, and sputter-coated with 12–15-nm gold. Specimens were examined with a Philips 501 microscope.

Results

Nerve cells began to adhere to the muscle fibers immediately after mixing, and within a few minutes most of the cells were engaged in large co-aggregates consisting of groups of muscle fibers held together laterally by nerve cells (Fig. 2). The time-

Figure 1. Filter well apparatus used to separate nerve–muscle aggregates from unbound nerve cells. The basic unit (lower left) consists of a 46-μm screen disc mounted with a plastic sleeve at the end of a glass tubing segment. The aggregates in each well are washed by repeated dipping in a dish of medium.

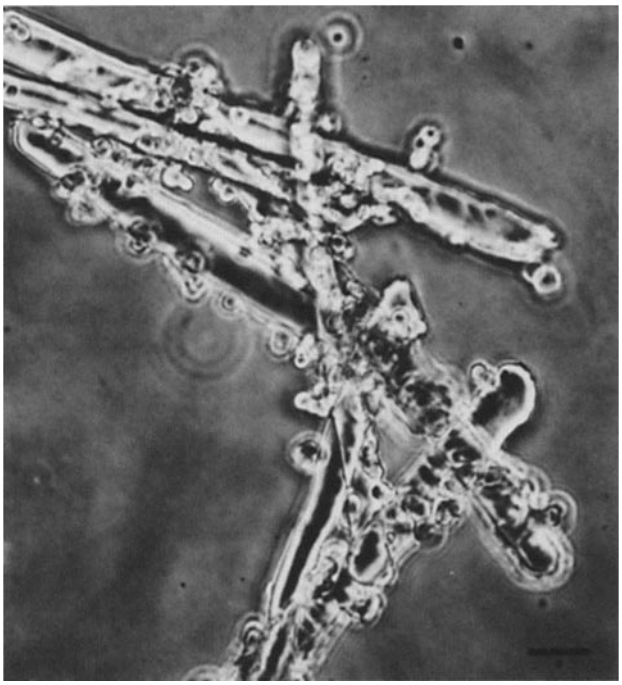

Figure 2. Phase-contrast micrograph of muscle fibers and PC-12 cells 10 min after mixing. Both cell types were initially monodisperse but have formed large co-aggregates in which groups of muscle fibers are bound together by attached PC-12 cells. Bar, 100 μm.

response showed that nerve cells adhered to the fibers as soon as mixing occurred, without a lag period (Fig. 3). Adhesion was complete by ~8 min with PC-12 cells and ~10–12 min with spinal cord cells. The adhesion was resistant to moderate trituration and centrifugation by 10–20 min after mixing, although the aggregates could be dissociated by vortexing.

The surface morphology of the two cell types was examined with the scanning electron microscope. The muscle fibers were covered with a smooth-surfaced basal lamina that reflects the underlying cross-striations of the sarcomeres (10; Fig. 4). An occasional collagen fibril remained from the reticular lamina external to the basal lamina. In contrast, the nerve cells were rough-surfaced and covered with many microappendages including folds, filopodia, and blebs. The two cell types appeared to be connected in places by fine filaments (Fig. 4). There was no obvious preferential adhesion of nerve cells to the motor end plate region of the fibers.

Cultivation of the co-aggregates for several days resulted in outgrowth of neurites from the nerve cells, thus confirming the neuronal identity of the spinal cord cells (Fig. 5). The neurites grew along the muscle fibers and often induced the formation of small patches of acetylcholine receptors on the surface of the muscle fibers (Fig. 6). Many of the nerve-induced acetylcholine receptor patches on the fibers were distant from the original site of the end plate near the center of the fibers. Isolated single muscle fibers retain high concentrations of acetylcholine receptors and acetylcholinesterase at the motor end plate (3, 9), but the acetylcholine receptor patches disappeared from the end plates after 2–3 d in vitro and did not reappear when the fibers were cultured in the absence of nerves. Spontaneous contractions occurred in most fibers, both in the presence and absence of nerves, and in some preparations twitching occurred even during dissociation of the fibers.

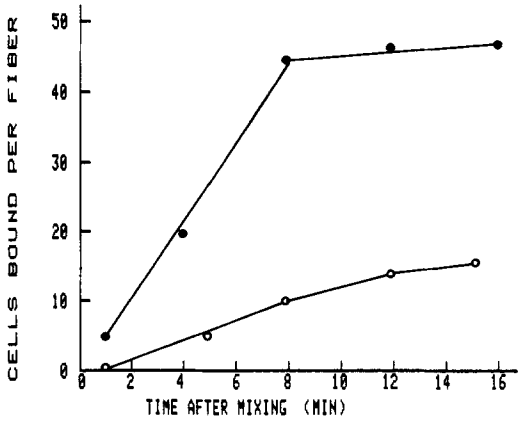

Figure 3. Time-response of adhesion of nerve cells to single muscle fibers. Labeled PC-12 cells (●) or rat spinal cord cells (○) were added to fibers at zero time and samples washed at intervals to remove unbound cells. Nerve–muscle aggregates were counted to determine nerve cells bound per fiber.

Several other types of cells were tested to determine the specificity of adhesion to muscle fibers. Spinal cord cells and PC-12 cells adhered to muscle rapidly to form large co-aggregates, while spinal ganglion cells adhered to a somewhat lesser extent. Other cells tested including brain cells, Schwann cells, and neuroblastoma cells, did not adhere at all (Table I).

Since the muscle fibers were prepared by enzymatic treatment, the adhesion of nerve cells might be an artifact of dissociation. To test this, single muscle fiber segments were prepared by mechanical dissection as described previously (7). PC-12 cells adhered to these fibers also in comparable numbers, but further study was precluded by the difficulty in obtaining these fibers. Nerve cells did not exhibit rapid adhesion to bundles of adult muscle fibers nor to myotubes formed in monolayer culture from rat embryo myoblasts.

Inhibition of Adhesion

The effect of various saccharides on adhesion was determined by adding the test substance at the time the cells were mixed together. Adhesion was measured by counting the number of [^{3}H]L-leucine-labeled nerve cells bound to a standard number of muscle fibers after removing unbound cells by filtration through nylon screen (see Materials and Methods). Of a variety of compounds tested, only *N*-acetylneuraminic acid (NANA) or mucin blocked adhesion (Table II). Both NANA and mucin showed similar inhibitory dose-response curves (Fig. 7). Inhibition of adhesion by NANA or mucin did not result from toxicity since cells pretreated with inhibitors adhered normally after washing thoroughly with medium. To determine whether the inhibition produced by mucin results from its sialic acids content, pig gastric mucin was fractionated into sialic acids–rich and sialic acids–poor components (Fig. 8). Only the fraction rich in sialic acids blocked adhesion. Removal of most of the sialic acids from the purified mucin by neuraminidase or acid hydrolysis reduced but did not eliminate the effect of mucin in blocking adhesion (Table II).

Efficiency of Adhesion

Nerve cell adhesion to muscle fibers was measured over a range of nerve cell concentrations to determine the dose-

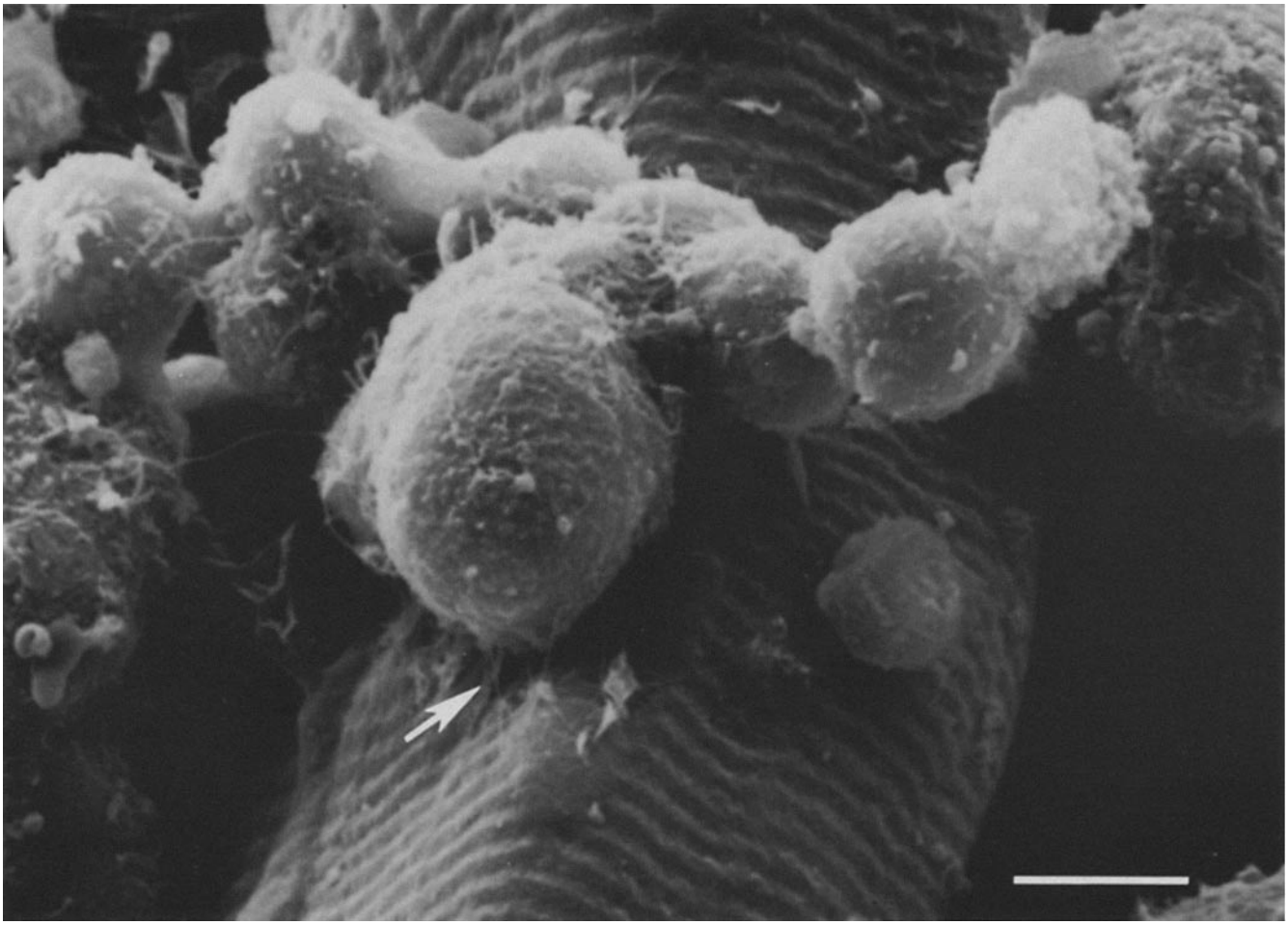

Figure 4. Scanning electron micrograph of PC-12 cells bound to a muscle fiber 10 min after mixing. Note the fine filaments (arrow) that appear to join the two cell types together. The PC-12 cells are somewhat clumped here but all quantitative adhesion studies were done with monodisperse cells. Bar, 5 μm.

response and fraction of cells bound. In these experiments a constant number of muscle fibers was mixed with variable numbers of nerve cells for 15 min to ensure complete adhesion, then unbound cells were removed by filtration and the co-aggregates were counted. Binding of PC-12 cells to fibers was much more efficient than was binding of spinal cord cells (Fig. 9). About 50% of the PC-12 cells bound to fibers, and the binding efficiency was linear over a wide range of cell concentrations to a maximum of ~100 cells bound per fiber. Spinal cord cell binding was also linearly related to cell input but only ~10% of the cells, from both rat and chick cords, bound to muscle fibers. With the rat spinal cord cells a plateau of adhesion was reached at an input of ~100 cells per muscle fiber, and addition of more cells did not increase the number bound to muscle fibers.

Properties of Adhesion

Various properties of nerve–muscle adhesion were studied to learn more about the reaction (Table III). Cells did not adhere when mixed at 4°C for up to 30 min, but promptly formed co-aggregates when warmed to room temperature. Once adhesion occurred, the co-aggregates did not dissociate when cooled to 4°C with mixing. Adhesion occurred well in Ca^{++}-free medium and was not blocked by pretreatment of both cell types with cytochalasin B or with azide and 2-deoxyglucose, inhibitors of energy metabolism. Adhesion was prevented if the fibers were first treated with Pronase, which removes the basal lamina from muscle fibers (6, 10).

Adhesion was partially inhibited by adding a crude membrane preparation from PC-12 cells (Table III). The membrane preparation contained only 20% of the total protein in the low speed supernatant of broken cells (see Material and Methods) but had virtually all the inhibitory activity.

To determine whether the binding activity on each cell type is qualitatively equivalent, cells were pretreated separately with either mucin or neuraminidase before mixing (Table IV). Since mucin is a large molecule with multiple oligosaccharide groups, it seemed likely that residual mucin might be retained on the cell surface long enough to block adhesion when cells were mixed in fresh medium. We found that mucin pretreatment of muscle fibers, but not nerve cells, abolished adhesion. If the adhesion assay was delayed after mucin pretreatment, the inhibition was diminished, presumably owing to eventual dissociation of the mucin. Prior digestion with neuraminidase was effective in preventing adhesion when used on nerve cells but not on muscle fibers. These results suggest that two different activities are involved in nerve–muscle adhesion: a

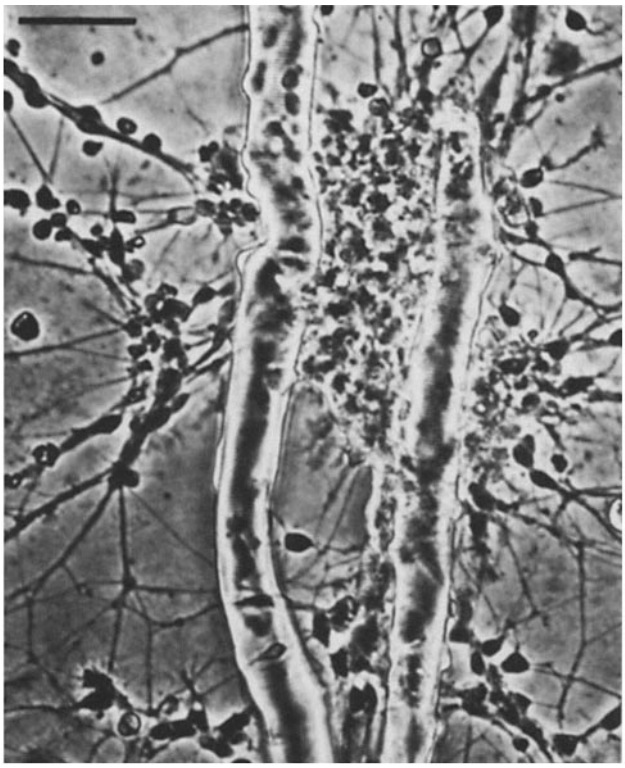

Figure 5. Phase-contrast micrograph of 3-d-old culture of nerve–muscle co-aggregates showing extensive neuritic outgrowth. The aggregates were attached to a collagen-coated coverslip by low speed centrifugation. Bar, 100 μm.

sialic acids–rich determinant on the nerve cells and a recognition site on the muscle fibers.

Discussion

The results reported here show that PC-12 cells and spinal neurons rapidly bind to single skeletal muscle fibers isolated from adult rats. This binding is not nonspecific, for a variety of other cell types tested have no affinity for the muscle fibers. Inhibition studies indicate that adhesion is mediated by a heterotypic mechanism in which a receptor located on the surface of the muscle fiber binds to a ligand rich in sialic acids on the nerve cell. NANA is the only simple sugar tested that blocks adhesion, but purified mucin is a better inhibitor. Thus, it is likely that the structural requirements of the active site are more complex than just NANA. Indeed, some compounds with high NANA content, such as orosomucoid and fetuin, failed to interfere with adhesion. Also, removal of most of the sialic acids from mucin did not completely eliminate inhibition.

The results of quantitative experiments on binding efficiency show that only a fraction of the nerve cells adhere to muscle fibers: ~50% of PC-12 cells and 10% of spinal cord cells. This incomplete binding does not result from inadequate time for adhesion or gradual decrease in collision frequency since the time-response curve (Fig. 3) reached a sharp plateau when there were still many unbound cells. At maximum adhesion, the aggregates still had space on the fibers for more nerve cells, and electron microscopic examination did not reveal obvious membrane fragments or vesicles on the fibers.

Figure 6. Fluorescent micrograph of 7-d-old culture prepared as in Fig. 5 and reacted with fluorescein isothiocyanate–labeled alpha-bungarotoxin to stain acetylcholine receptors. Several patches of staining are present on the muscle fiber. Fibers cultured without nerve showed no toxin binding at 7 d. Bar, 50 μm.

*Table I. Specificity of Adhesion to Muscle Fibers**

Adhesion
Rat embryo spinal cord
Rat embryo spinal ganglion
Chick embryo spinal cord
Rat pheochromocytoma PC-12
No adhesion
Rat embryo fibroblasts
Rat embryo liver
Rat embryo Schwann cells
Rat embryo cerebellum
Rat embryo cerebral cortex
Human neuroblastoma NGP
Human neuroblastoma NMB
HeLa
Rat erythrocytes
Rabbit erythrocytes

* The test cells were mixed with dissociated muscle fibers for 10 min, then the unbound cells were removed by sedimentation, and the fibers were scored by microscopy. The cells scored as negative bound at less than one cell per fiber, while adhesion-positive cells bound more than 10 cells per fiber. The spinal ganglion cells bound to fibers at lower levels than the other cells in this group, but quantitative measurements were not made.

*Table II. Effect of Sugars and Glycoproteins on Nerve-Muscle Adhesion**

	Concentration	Nerve cells bound per fiber (% control)
Sugar		
Galactose	100 mM	106 ± 14‡
Lactose		98 ± 3
Fucose		102 ± 9
Methyl-mannoside		91 ± 11
Mannose-6-phosphate		93 ± 8
N-acetyl-galactosamine		68 ± 21
N-acetyl-glucosamine		80 ± 5
N-acetyl-neuraminlactose		112 ± 8
NANA		3 ± 1
	50 mM	28 ± 3
Glycoprotein		
Salivary mucin	1.0 mg/ml	4 ± 0.7
Gastric mucin		6 ± 2
Gastric mucin-neuraminidase§		48 ± 6
Fetuin-Spiro method		102 ± 5
Fetuin-Deutsch method		98 ± 12
Orosomucoid		180 ± 24
Yeast mannan		98 ± 4
Hyaluronic acid		105 ± 2
Chondroitin sulfate		103 ± 14
Heparin		87 ± 7

* Labeled PC-12 cells were mixed with muscle fibers for 10 min under the conditions listed, and unbound cells were removed by filtration. Bound cells were determined by scintillation counting and expressed as percent untreated control mixtures.

‡ Mean ± standard error.

§ Mucin treated with 3 mg/ml neuraminidase for 12 h at 37°C, then fractionated on a Sephadex G-25 column. This removed ~80% of the sialic acids from the mucin.

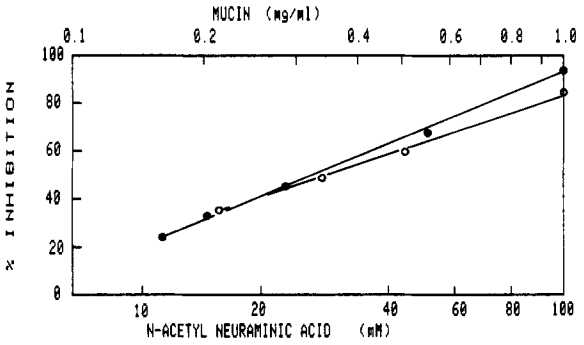

Figure 7. Inhibitory dose-response of adhesion to NANA (●) and gastric mucin (○). Labeled PC-12 cells were mixed with muscle fibers in the presence of the inhibitors for 10 min, then unbound cells were washed away and the aggregates were counted.

The adhesion sites may have been occupied, however, by soluble ligand shed from the nerve cells. This would not be visible in the electron microscope. Shedding of ligand is supported by the dose-response curve for rat spinal cord cells which reaches a plateau at relatively low cell number per fiber.

The basis of the difference in binding efficiency between the PC-12 cells and spinal cord cells is not clear. In the case of the spinal cord, adhesion may be restricted to the motor neuron population which represents only a small fraction of the total dissociated cells. Since the PC-12 cells were proliferating, they were heterogeneous with respect to the cell cycle, and this may influence synthesis or exposure of adhesive sites. Also, soluble ligand, as discussed above, could be a factor in binding efficiency.

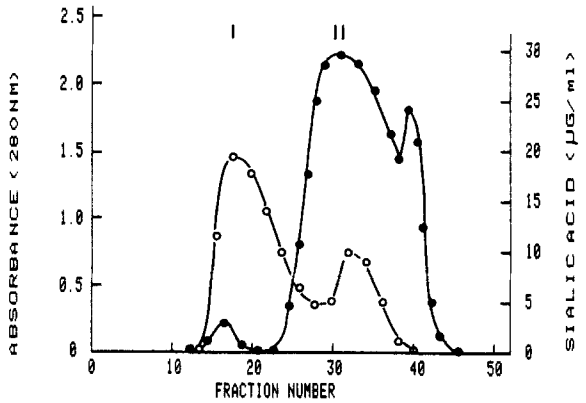

Figure 8. Fractionation of gastric mucin on a Sephacryl S-300 column into sialic acids-rich (peak *I*) and sialic acids-poor (peak *II*) components. Protein was measured by absorbance at 280 nm (●) and sialic acids were measured by the periodate-resorcinol reaction (○). When tested in the quantitative adhesion assay at various protein concentrations, peak *I* material blocked adhesion (96% inhibition) at 25 μg/ml, the lowest concentration tested, while peak *II* material produced 50% inhibition of adhesion at 1,000 μg/ml.

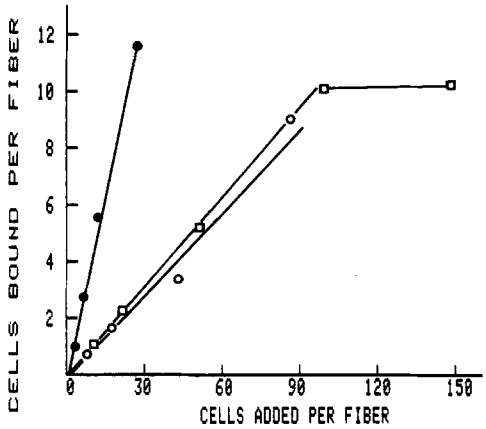

Figure 9. Dose-response of nerve cell binding to single muscle fibers. Various numbers of labeled PC-12 cells (●), chick spinal cord cells (○), or rat spinal cord cells (□) were added to aliquots of muscle fibers and mixed for 15 min to ensure complete binding. Unbound cells were removed by washing, and the aggregates were counted to determine cells bound per fiber.

The kinetics of binding and the effects of drugs on adhesion suggest that attachment of nerve to muscle requires few active processes at the cell surface. Nerve cells bind to muscle as soon as contact is made, with no detectable lag period, and maximum adhesion is complete in <10 min with PC-12 cells. Adhesion is not affected by pretreating cells with inhibitors of glycolysis and oxidative energy production or by cytochalasin B, which interferes with actin polymerization. Taken together, this behavior suggests that nerve-muscle attachment occurs through sites that are exposed on the cell surface before cell contact. Based on these characteristics, the mechanism of nerve-muscle adhesion resembles cell attachment mediated by concanavalin A but not by fibronectin (15).

*Table III. Effect of Various Treatments on Nerve–Muscle Adhesion**

Treatment	Nerve cells bound per fiber (% control)
Cytochalasin B, 10 μg/ml§	94 ± 3‡
Cytochalasin B, 1 μg/ml	103 ± 29
2-Deoxyglucose + azide‖	99 ± 8
Mix at 4°C	6 ± 0.5
Mix at room temperature, then 4°C	90 ± 3
Calcium-free medium¶	79 ± 3
Pronase-treated muscle fibers	5 ± 1
Membrane fraction from nerve cells**	29 ± 2
Non-membrane fraction from nerve cells	88 ± 2

* Labeled PC-12 cells and fibers were mixed as described in Table II.

‡ Mean ± standard error.

§ Nerve cells and fibers were pretreated for 30 min before mixing.

‖ Cells pretreated 30 min with 100 mM 2-deoxyglucose plus 125 μg/ml sodium azide before mixing.

¶ Cells were mixed in Ca^{++}-free PBS with 10% dialyzed serum.

** 0.5 mg/ml protein from PC-12 membranes prepared as described in Materials and Methods.

*Table IV. Pretreatment of Cells with Mucin or Neuraminidase**

Cell treated	Cells bound per fiber	% Control
	Mucin	
None	66 ± 4	
Nerve	63 ± 7	96
Muscle	3 ± 0	4
Both	2 ± 0	3
	Neuraminidase	
None	58 ± 1	
Nerve	4 ± 2	7
Muscle	56 ± 17	95
Both	5 ± 1	9

* PC-12 cells labeled with [^{3}H]L-leucine or muscle fibers were treated with either 1 mg/ml mucin or 0.2 mg/ml neuraminidase as described in Materials and Methods, washed, and mixed for 10 min. Unbound cells were removed by filtration, and the aggregates were counted. The percent control values shown for neuraminidase treatment are in reference to the enzyme blank using boiled neuraminidase.

Since the basal lamina is the outermost surface of the muscle fiber, to which nerve cells adhere, the receptor activity may be a component of the basal lamina. Both laminin and fibronectin are present in the muscle fiber basal lamina (45) and both have cell binding domains (49) and the ability to agglutinate erythrocytes (31, 41). Laminin has been identified by immunofluorescent staining in the basal lamina of the single fibers used in this study (10). Agglutination of trypsin- and glutaraldehyde-treated rabbit erythrocytes by laminin requires calcium and is inhibited by mucin and heparin but not by NANA (41). Fibronectin-mediated erythrocyte agglutination also requires calcium and is inhibited by galactosamine but not by NANA (32). In contrast, nerve–muscle adhesion does not require calcium and is blocked by mucin and NANA but not by heparin or galactosamine (Tables II and III). Furthermore, purified Schwann cells adhere to laminin-coated surfaces (38) but do not attach to muscle fibers (Table I).

N-CAM is reported to be an integral membrane glycoprotein (17) and as such would not be expected to play a role in nerve–muscle adhesion that takes place across a basal lamina. Nevertheless, N-CAM is rich in sialic acids and has been implicated in adhesion of neurites to embryonic myotubes (22). The homotypic binding (N-CAM → N-CAM) thought to be mediated by N-CAM does not require calcium and is not prevented by removal of sialic acids with neuraminidase (17). Also, N-CAM cannot be detected in normal adult rat muscle using a polyclonal antibody that recognizes several types of N-CAMs (16). It seems unlikely therefore, that the nerve–muscle adhesion reported here results from N-CAM → N-CAM interaction. Spinal cord cells contain N-CAM on their surface, however (48), and it is possible that a receptor on the muscle fiber basal lamina recognizes the sialic acids–rich moiety of N-CAM on the neurons. More detailed comparisons with previously characterized cell surface components await isolation of the adhesion molecules from nerve and muscle cells.

If the adhesion mechanism described here is of general importance in nerve–muscle interaction, it is surprising that previous studies involving addition of nerve cells to muscle cultures have not reported this phenomenon. We observed that nerve cells bind rapidly to single adult muscle fibers prepared by enzymatic disaggregation or mechanical dissection but not to bundles of adult muscle fibers or to monolayers of myotubes. The clue to this behavior may lie in the nature of the muscle surface presented to the nerve cells. The single fibers prepared enzymatically (10) or mechanically (7) are covered with a basal lamina but have little or no remaining collagen fibrils of the reticular lamina external to that. Mechanical dissection of single fibers separates the fiber from the connective tissue matrix at the interface between the basal lamina and the reticular lamina (8). Thus, nerve cells can make direct contact with the muscle fiber basal lamina in these cases. In contrast, muscle fibers removed as a bundle are covered with a continuous meshwork of reticular collagen fibrils (28; Bischoff, R., unpublished observations) and well-differentiated monolayer cultures are overlain with a collagen and proteoglycan layer (1, 47). In the animal, neuronal growth cones would be able to contact and adhere to the basal lamina surface of the fiber by insinuating microappendages through the collagen meshwork of the reticular lamina.

Interaction of the nervous and muscular systems occurs across the basal lamina of the synapse, yet little is known about the function of this layer. There are several possibilities. Since patches of basal lamina material are present from the earliest contact between nerve and muscle both in vivo (14) and in vitro (2, 5), the basal lamina may contain specific cell recognition determinants that enable the growth cone to sense its target. Also, recent experiments have shown that the basal lamina of the mature synapse contains information that allows ingrowing nerves to return to their original sites after denervation (37, 46). In view of these results, it is surprising that the dissociated nerve cells used in the present study had no special affinity for the junctional region but adhered along the length of the fiber. This may reflect the existence of several levels of adhesion. The first, as described here, may bind the nerve terminal to any part of the muscle fiber and allow it to move along the fiber until contact is made with the end plate where a second level of adhesion may govern the final synaptic differentiation. Finally, the basal lamina probably mediates mechanical attachment between nerve and muscle at all stages

of development. There is no information on the adhesive characteristics of the neuromuscular junction, but Kanwar and Farquhar (30) have recently shown that the glomerular basal lamina can be detached from the endothelium and podocytes by neuraminidase, suggesting the importance of sialic acids in attachment of these cells to the basal lamina.

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