# Cell-Substratum Adhesion in Embryonic Chick Central Nervous System Is Mediated by a 170,000-mol-wt Neural-specific Polypeptide

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ABSTRACT Embryonal chick neural retina cells release into the culture medium a complex of proteins and glycosaminoglycans, termed adherons, that promote cell to substratum adhesion. A monoclonal antibody ($C_1H_3$) blocks adheron-mediated cell to substratum adhesion and specifically binds to a 170,000-mol-wt protein present in retinal adherons (Cole, G. J., and L. Glaser, 1984, *J. Biol. Chem.*, 259:4031–4034). The 170,000-mol-wt protein also can be identified in embryonic chick brain and peripheral nervous tissue. In the neural retina, $C_1H_3$ also binds to a second antigen with a molecular weight of 140,000 that is absent in the brain. Embryonic brain, therefore, provides a source for the immunopurification of the 170,000-mol-wt protein. Brain adherons also contain the 170,000-mol-wt protein, and cell to substratum adhesion mediated by these adherons is blocked by the $C_1H_3$ monoclonal antibody. The 170,000-mol-wt protein in the brain is therefore functionally identical to that in the retina. To demonstrate that adheron-mediated cell to substratum adhesion is caused by cell binding to the 170,000-mol-wt protein, we showed that (*a*) protease digestion, but not glycosaminoglycan hydrolase digestion of adherons, blocked their ability to bind cells to substratum; (*b*) the immunopurified 170,000-mol-wt protein blocks adheron-mediated cell to substratum adhesion; and (*c*) cells can bind to immunopurified 170,000-mol-wt protein bound to glass surfaces.

Adhesive interactions between cell types in the developing nervous system are likely to be dependent on several distinct processes. A primary mode of interaction occurs between neurons and is in part mediated by the neural cell adhesion molecule, N-CAM (1).[1] Molecules participating in neuron-neuron interactions that are immunochemically or functionally related to N-CAM have also been described in the nervous system (2–4). Grumet et al. (5) have recently identified a molecule distinct from N-CAM that participates in adhesion between neurons and glia. In addition to these types of cell-cell adhesion, extracellular molecules have been implicated in cell adhesion processes. Macromolecules that comprise the extracellular matrix provide a substratum that permits the adhesion and migration of developing cells, and thus probably play a key role in development. Extracellular molecules that are possible candidates in cell-substratum adhesion have been identified in cultured muscle cells (6, 7) and fibroblast-like cells (8, 9). Characteristic of these molecules is their release from the cells into the culture medium, and their promotion of cell-substratum adhesion when used to coat plastic culture dishes. Extracellular macromolecules that are released by non-neural cells and permit cell-substratum adhesion of neural cells have also been described (10). In addition, a complex of proteins and glycosaminoglycans, termed adherons,[2] has been isolated from the culture medium of embryonic chick neural retina cells and has specifically been shown to mediate cell-substratum adhesion of these neural cells (11). Neural retinal cells adhere specifically to plastic dishes coated with retinal

[1] *Abbreviations used in this paper*: buffer A, 8 g NaCl, 0.2 g KCl, 0.2 g $KH_2PO_4$, 0.15 g $Na_2HPO_4$/liter (pH 7.4); DME, Dulbecco's minimal essential medium; EBSS, Earle's balanced salt solution; N-CAM, neural cell adhesion molecule; NP-40, Nonidet P-40.

[2] The term adheron is used operationally to describe particulate material prepared by the method of Schubert et al. (11). It is not meant to imply that this material represents a defined macromolecular aggregate of constant composition.

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adherons, and adherons that show specificity for the cell of origin have been isolated from muscle cells (6, 7).

A monoclonal antibody, termed $C_1H_3$, was prepared in our laboratory using intact embryonic chick neural retina cells as an immunogen. In extracts of the neural retina, this monoclonal antibody reacts with two distinct developmentally regulated polypeptides with molecular weights of 170,000 and 140,000 (12). The 170,000-mol-wt $C_1H_3$ polypeptide is released from cultured retina cells into the culture medium and is a component of adherons (13). The $C_1H_3$ antibody abolishes adheron-mediated cell to substratum adhesion of retinal cells, either when incubated with the adherons or with the cells before assay. This observation suggests that retinal cell binding to adherons is homophilic, i.e., mediated by like molecules—one on the cell, the other in the adheron. Antibody blocking experiments are potentially misleading in that the blocking effect of the antibody could be due to steric effects. If the adheron-mediated cell to substratum adhesion is due to cell binding to the 170,000-mol-wt protein, one can expect the following: (*a*) soluble 170,000-mol-wt protein will block adhesion; (*b*) cells will bind to 170,000-mol-wt proteins immobilized on an inert support; and (*c*) cell to adheron binding will be sensitive to digestion with proteolytic enzymes and insensitive to treatment with glycosaminoglycan hydrolases. Our experiments provided evidence that these predictions in fact pertain to retinal cell to substratum adhesion.

We purified the 170,000-mol-wt protein from detergent extracts of embryonal chick brain by immunoabsorption. The brain is the preferred source for this particular protein since the 170,000-mol-wt immunoreactive protein, but not the 140,000-mol-wt immunoreactive protein, is present in brain (12). To validate the use of brain as a source of this protein, we showed that brain adherons also contain the 170,000-mol-wt protein and are functionally indistinguishable from retinal adherons.

## MATERIALS AND METHODS

*Preparation and Screening of Monoclonal Antibodies:* Preparation of the $C_1H_3$ monoclonal antibody has been described previously (12). It was obtained by the immunization of Sprague-Dawley rats with dissociated day 7 retinal cells and the fusion of rat spleen cells with the mouse myeloma Sp 2/0 cell line. The antibody was isolated by precipitation of immunoglobulins from culture medium with saturated ammonium sulfate, followed by chromatography on a CM Affi-Gel blue column to remove albumin.

The monoclonal antibody to N-CAM was obtained from Dr. David I. Gottlieb (Washington University). It was prepared by immunizing BALB/c mice with mechanically dissociated day 9 retinal cells, and fusing the spleen cells with Sp 2/0 myeloma cells (14). The anti-N-CAM antibody used in these studies was obtained by injecting BALB/c mice with hybridoma cells and isolating ascites fluid containing the immunoglobulin.

*Isolation of Adheron Complexes:* Adherons were isolated from dissociated retina or brain cells as described by Schubert et al. (11), with some modifications. Our primary modification was the mechanical dissociation of the embryonic tissues, as opposed to trypsin dissociation, before culturing. Embryonic day 11 retinas or day 12 brains were mechanically dissociated with a fire-polished Pasteur pipette and incubated for 18 h at 37°C in serum-free Dulbecco's minimal essential medium (DME) that contained transferrin, insulin, progesterone, and putrescine (15). Adherons were isolated from conditioned medium as described by Schubert et al. (11). Briefly, conditioned medium was centrifuged 5 min at 1,000 *g* to remove cells, and then centrifuged at 12,000 *g* for 30 min to remove cell debris. The supernatant fluid was then centrifuged 3 h at 100,000 *g*, the supernatant was discarded, and the adheron pellet was washed twice by centrifugation with Earle's balanced salt solution (EBSS) (Gibco Laboratories, Grand Island, NY).

The presence of the $C_1H_3$ antigen in isolated adherons was determined by two separate methods. The first involved dot-blotting of adheron protein onto nitrocellulose (16), followed by incubation with $C_1H_3$ monoclonal antibody and horseradish peroxidase-conjugated goat anti-rat IgG (Cappel Laboratories, Cochranville, PA). Antigen was then visualized by reaction of the filter with 3,3′-diaminobenzidine. The second method used to identify the $C_1H_3$ antigen in isolated adherons entailed the isolation of adherons from cultures of dissociated retina or brain cells that had been labeled overnight in methionine-free DME containing 10% dialyzed fetal calf serum with 10 $\mu$m L-methionine and 20 $\mu$Ci/ml of [$^{35}$S]methionine (translation grade) (New England Nuclear, Boston, MA). Adheron complexes were solubilized in immunoprecipitation buffer (150 mM NaCl, 20 mM $Na_2HPO_4$, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM benzamidine-HCl, 0.3 mM phenylmethylsulfonyl fluoride, 1 mM L-methionine [pH 7.5]) as described previously (12) and analyzed by PAGE. For immunoprecipitation of the $C_1H_3$ antigen, solubilized adherons were incubated overnight at 4°C with *Staphylococcus aureus* cells (Calbiochem-Behring Corp., San Diego, CA) that had been coated with goat anti-rat whole serum and an excess of $C_1H_3$ monoclonal antibody. Precipitated $C_1H_3$ antigen was solubilized in electrophoresis sample buffer, heated to 100°C for 5 min, and separated on 5% SDS polyacrylamide gels.

*Assay of Cell-Substratum Adhesion:* Cell-substratum adhesion was assayed, with slight modifications, according to published methods (11). Day 11 retina cells or day 13 brain cells were mechanically dissociated and labeled 2–4 h with 10 $\mu$Ci of [$^{35}$S]methionine as outlined previously. Labeled cells were washed twice with EBSS containing 0.2% albumin, and 0.2-ml aliquots were pipetted into 35-mm plastic petri dishes, that contained 2 ml of the same medium and that had been coated with 30–50 $\mu$g of adheron protein. Dishes were incubated 1 h at 37°C and swirled 10 times to dislodge cells that adhere weakly to the dish. Medium was then aspirated, attached cells were dissolved in Triton X-100, and isotope content was measured. Under the conditions employed in our studies, the assay of cell-substratum adhesion represents the measurement of adhesive rate because the binding of cells to substratum is still rising after the 60-min incubation.

To examine the effect of $C_1H_3$ monoclonal antibody (or other monoclonal antibodies) on cell-substratum adhesion, we incubated 0.2-ml aliquots of labeled cells for 30 min at 4°C, with 150 $\mu$g of protein of an immunoglobulin fraction isolated as described previously (13). Cells were then washed with EBSS containing 0.2% albumin, and their ability to attach to adheron-coated dishes was assessed.

To find out if immunopurified 170,000-mol-wt $C_1H_3$ polypeptide could promote adhesion, we coated glass vials with the antigen; subsequently, these vials were used to measure the rate of cell-substratum adhesion. To attach immunopurified antigen to the surface of glass vials, we used the protocol of Gottlieb and Glaser (17). Briefly, we washed standard glass scintillation vials with nitric acid and then treated with a 10% solution of $\gamma$-aminopropyltriethoxysilane in toluene for 2 h at room temperature. The vials were washed with fresh toluene and air dried. A 1% aqueous solution of glutaraldehyde was then added to the vial and left for 1 h at room temperature. After thorough washing with distilled water and air drying, the vials were incubated overnight with total adheron protein, immunopurified antigen, or bovine serum albumin.

*Immunoaffinity Purification of $C_1H_3$ Antigen:* Brains were removed from 80 day-14 chick embryos and homogenized in 20 ml of calcium-magnesium–free medium containing 0.3 mM phenylmethylsulfonyl fluoride and 1 mM EDTA. The homogenate was centrifuged for 15 min at 12,000 *g* and the pellets were resuspended in 2.25 M sucrose in 8 g of NaCl, 0.2 g of KCl, 0.2 g of $KH_2PO_4$, 0.15 g of $Na_2HPO_4$/liter (pH 7.4) (buffer A), according to published methods (18). The resuspended pellets were transferred to ultracentrifuge tubes and then overlaid with 0.8 M sucrose in buffer A. After centrifugation for 1 h at 100,000 *g*, the material at the interface between the two sucrose solutions was collected, washed twice with buffer A, and used for immunopurification of $C_1H_3$ antigen.

Before immunopurification, day 14 brain membranes were solubilized by overnight stirring in buffer A containing 1 mM EDTA, 0.5% Nonidet P-40 (NP-40), (pH 8.2). $C_1H_3$ monoclonal antibody (5 mg/ml) was then coupled to CNBr-activated Sepharose 4B. The immunoaffinity matrix was then incubated by end-over-end shaking for 1.5 h with the NP-40 extract of brain membranes. The immunoadsorbent was washed twice with solubilization buffer as described by Hoffman et al. (18), and the immunoadsorbent was collected in a column and washed with three column volumes of buffer A containing 1 mM EDTA, 0.5% NP-40. $C_1H_3$ antigen was eluted with 2.5 column volumes of buffer A containing 1 mM EDTA, 0.5% NP-40, 0.05 M diethylamine (pH 11.5). The eluate was neutralized with 0.1 vol of 1.0 M potassium phosphate (pH 7.0) and detergent was removed by chromatography on a column of Extracti-gel D (5 ml of eluate/ml gel) (Pierce Chemical Co., Rockford, IL). The eluate was then dialyzed overnight against 500 volumes of $H_2O$ at 4°C, concentrated by centrifugation in an Amicon microconcentrator (Amicon Corp. Scientific Sys. Div., Danvers, MA), and stored frozen at −70°C.

*Enzymatic Treatment of Adheron Complexes:* The role of glycosaminoglycans in cell-substratum adhesion was determined by incubating culture dishes coated with retina adheron protein with 1.0 U of chondroitinase ABC (Sigma Chemical Co., St. Louis, MO), hyaluronidase (Sigma Chemical Co.) or heparitinase (Miles Laboratories, Elkhart, IN) in EBSS at 37°C for 30–60 min. Glycosaminoglycan breakdown was monitored by identically treating culture dishes in duplicate that had been coated with adheron protein obtained from retina cultures labeled with [$^{35}$S]sulfate (Amersham Corp., Arlington Heights, IL). After enzymatic treatment, the culture dishes were washed three times with EBSS that contained 0.2% albumin, and cell-substratum adhesion was measured.

The role of polypeptides in cell-substratum adhesion was determined by incubating adheron-coated dishes with 0.01% trypsin (Sigma Chemical Co.) or 0.01% Subtilisin BPN protease (Sigma Chemical Co.) in EBSS at 37°C for 30 min. Protein breakdown by enzyme treatment was estimated by identically treating culture dishes in duplicate that had been coated with [$^{35}$S]methionine-labeled adherons. Cell-substratum adhesion was subsequently assayed as outlined above.

## RESULTS

### *Brain Adherons Mediate Cell to Substratum Adhesion and Contain the 170,000-mol-wt Protein*

To examine the role of the 170,000-mol-wt $C_1H_3$ polypeptide in cell-substratum adhesion, it is first necessary to immunopurify the antigen using a monoclonal antibody affinity column. Since the $C_1H_3$ monoclonal antibody recognizes two distinct polypeptides with molecular weights of 170,000 and 140,000 in embryonic chick neural retina, but only reacts with the 170,000-mol-wt component in embryonic brain (12), a more feasible approach is to use embryonic chick brain tissue for the immunopurification of the 170,000-mol-wt protein. It was not known whether the brain 170,000-mol-wt protein was functionally identical to the retinal protein. To test this, we prepared brain adherons and used them to coat plastic dishes. The protocol for such experiments is shown in Fig. 1. The data in Fig. 2 show that brain adherons promote, in a dose-dependent manner, the adhesion of brain cells to the dish, and that this adhesion can be blocked with $C_1H_3$ antibody, but not with anti-N-CAM antibody (19). Fig. 3 illustrates that brain adherons and retinal adherons are functionally indistinguishable, in that both types of adherons are equally efficient in supporting the attachment of retinal cells or brain cells[3] to the substratum.

The polypeptide composition of brain and retinal adherons biosynthetically labeled with [$^{35}$S]methionine is shown in Fig. 4, lanes *a* and *b*. Although there are several differences in the composition of both types of adherons, both contain a 170,000-mol-wt polypeptide. The $C_1H_3$ antibody can be utilized to specifically immunoprecipitate this polypeptide from dissociated brain adherons (Fig. 4, lane *c*). N-CAM, which mediates cell to cell adhesion in many regions of the nervous system, is absent in adherons but present in the cells of origin. This is illustrated for retinal cells and retinal adherons in Fig. 5 and Fig. 4, lane *d*. Although these data suggest that N-CAM and the 170,000-mol-wt $C_1H_3$ protein are distinct and unrelated polypeptides, this cannot be stated with certainty. Recent evidence has indicated that some monoclonal antibodies to distinct cell adhesion molecules do exhibit cross-reactivity (5).

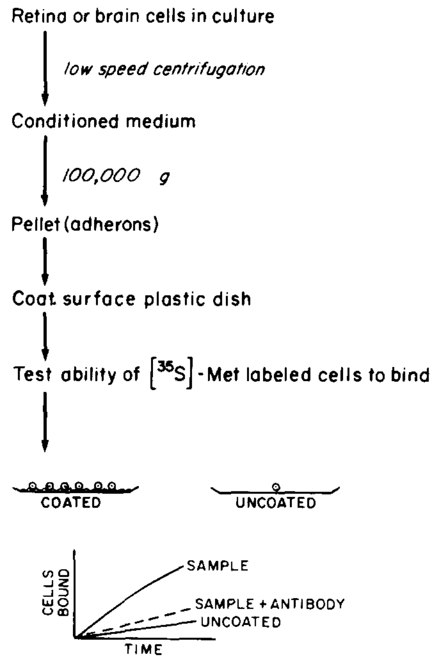


FIGURE 1 Schematic diagram of protocol for the isolation of adheron particles and the analysis of cell-substratum adhesion. Dissociated retinal or brain cells were incubated overnight at 37°C as described under Materials and Methods. The conditioned medium is then centrifuged at 100,000 *g* to sediment adheron particles. To assess cell-substratum adhesion, we coated plastic culture dishes with the resuspended adheron pellet and then incubated with [$^{35}$S]methionine-labeled retinal or brain cells. Cell to substratum adhesion was quantitated by dissolving bound cells in Triton X-100 and measuring isotope content. As illustrated in the graph, cells bind poorly to uncoated plastic but bind at increased rates to adheron-coated plastic. The role of specific macromolecules in cell to substratum adhesion can be examined by employing monoclonal antibodies to block adhesion.

It thus remains to be determined to what extent, if any, the 170,000-mol-wt $C_1H_3$ polypeptide is related to other cell adhesion molecules in nervous tissue.

We concluded that retinal and brain adherons are functionally indistinguishable, and that the brain-derived 170,000-mol-wt protein can be used to test whether cell to adheron binding is due to this polypeptide.

### *Effect of Immunopurified 170,000-mol-wt $C_1H_3$ Polypeptide on Cell-Substratum Adhesion*

Our observations, taken together with previous observations, indicated that embryonic chick brain and retinal cells employ a similar mechanism for cell-substratum adhesion, and that the mechanism of binding may be homophilic (i.e., binding required the interaction of like molecules on the cell surface and in the adheron complex), with the 170,000-mol-wt $C_1H_3$ polypeptide functioning as the adhesion molecule. We therefore investigated whether purified brain $C_1H_3$ antigen could disrupt cell-substratum adhesion. To isolate the 170,000-mol-wt antigen, embryonic day 14 brain membranes were solubilized in NP-40 and incubated with $C_1H_3$ monoclonal antibody covalently coupled to Sepharose-4B (18). Bound antigens were eluted with diethylamine (pH 11.5) and NP-40 was removed by chromatography on a detergent-affinity column and extensive dialysis. The purity of the immunopurified antigen was assessed by PAGE. The 170,000-

[3] We examined the binding of brain cells obtained from embryos of different ages to adheron-coated dishes obtained from 11-d-old embryonal brain cells that were maintained for 1 d in culture. Maximal adhesion is obtained with cells from 13-d-old embryos; these were used in all experiments. Cells from 11- or 15-d-old embryos adhere at about ½ the rate of day 13 brain cells in this assay.

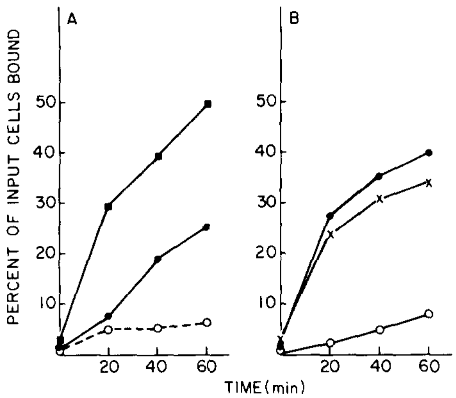


FIGURE 2 Stimulation of cell-substratum adhesion by brain adherons. Particulate material from conditioned medium of cultured brain cells was prepared as described. (*A*) 35 mm plastic Petri dishes were coated with either 30 $\mu$g (●) or 50 $\mu$g (■) of adheron protein. Dissociated day 12 brain cells that had been metabolically labeled with [$^{35}$S]methionine were added to the dishes and incubated at 37°C for the times indicated. Following incubation, dishes were swirled gently to dislodge cells that were weakly adherent, medium was aspirated, and bound cells were dissolved in Triton X-100 to measure isotope content. Binding of cells to uncoated plastic is indicated by open circles. (*B*) Effect of monoclonal antibodies on cell-substratum adhesion. Culture dishes were coated with 40 $\mu$g of adheron protein as described. Metabolically labeled day 12 brain cells were then added to the dishes following either no treatment (●), incubation with anti-N-CAM monoclonal antibody (×), or incubation with $C_1H_3$ monoclonal antibody (○). Cell-substratum adhesion was quantitated as described above. Anti-N-CAM monoclonal antibody (19) was obtained from Dr. D. I. Gottlieb, Washington University.

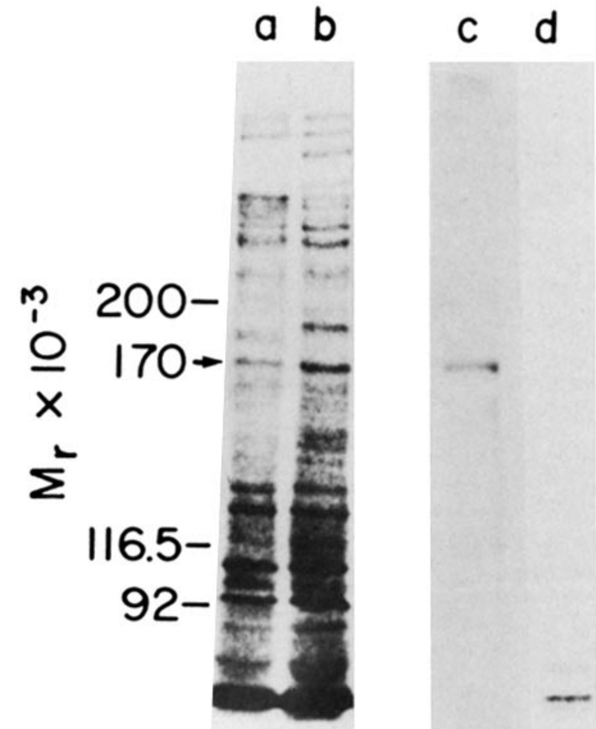


FIGURE 4 SDS PAGE of metabolically labeled adheron proteins. Cultures of embryonic day 11 retinal cells, or day 12 brain cells, were incubated overnight in Spinner culture at 37°C in the presence of [$^{35}$S]methionine as described under Materials and Methods. Aliquots containing similar amounts of radioactivity from retinal adheron or brain adheron pellets were then separated on 5% polyacrylamide gels. For immunoprecipitation (lanes *c* and *d*), adheron pellets were solubilized in detergent and incubated overnight at 4°C with *S. aureus* cells coated with goat anti-rat whole serum and $C_1H_3$ monoclonal antibody (lane *c*) or goat anti-mouse whole serum and anti-N-CAM monoclonal antibody (lane *d*). Immunoprecipitated polypeptides were separated on 5% polyacrylamide gels. (Lane *a*) Total polypeptides contained in brain adheron preparation. The 170,000-mol-wt polypeptide is denoted by the arrow. (Lane *b*) Total polypeptides from retina adheron preparations. (Lane *c*) Immunoprecipitation of the 170,000-mol-wt polypeptide from brain adherons using the $C_1H_3$ monoclonal antibody. (Lane *d*) Immunoprecipitation of retina adherons using anti-N-CAM monoclonal antibody. Note the absence of N-CAM, as measured by immunoprecipitation, in the adheron preparation.

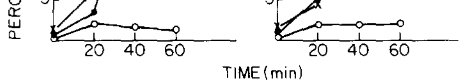


FIGURE 3 Adhesion of retinal and brain cells to their homologous or heterologous adherons. Dissociated day 11 retinal cells or day 12 brain cells were incubated for 18 h in serum-free DME as described under Materials and Methods. 35-mm plastic petri dishes were then coated with 30 $\mu$g of either day 12 retinal or day 13 brain adheron protein. Adhesion of metabolically labeled day 12 retinal or day 13 brain cells to these adheron-coated dishes was measured at the times indicated. (*A*) Adhesion of labeled neural cells to retina adherons. (*B*) Adhesion of labeled neural cells to brain adherons. (●) Day 12 retinal cells; (×) day 13 brain cells; (○) binding of neural cells to uncoated plastic.

mol-wt $C_1H_3$ polypeptide is the predominant polypeptide detected by staining with Coomassie Blue (Fig. 6), although a faint band at the molecular weight of 43,000 (presumably actin) was also detected. The band migrating at 170,000-mol-wt was confirmed as the $C_1H_3$ polypeptide by immunoblotting. When partially purified antigen is transferred to nitrocellulose and incubated with the $C_1H_3$ antibody, a pronounced band at the molecular weight of 170,000 was detected (data not shown). However, a minor immunoreactive component that is stained after incubation with only secondary antibody (and thus is probably representative of the IgG heavy chain) was also observed (data not shown).

Incubation of dissociated retinal cells with the purified 170,000-mol-wt protein resulted in a disruption of cell-substratum adhesion, which is dependent on the amount of purified polypeptide added (Fig. 7). When 10 $\mu$g of the partially purified 170,000-mol-wt protein were added to the assay, the inhibition of adhesion approached that observed with the $C_1H_3$ monoclonal antibody. In contrast, incubation of retinal cells with similar amounts of total brain protein that has been processed as described for the $C_1H_3$ antigen had little effect on cell-substratum adhesion. These results supported the proposal that adheron-mediated adhesion may occur by a homophilic mechanism involving the 170,000-mol-wt $C_1H_3$ polypeptide.

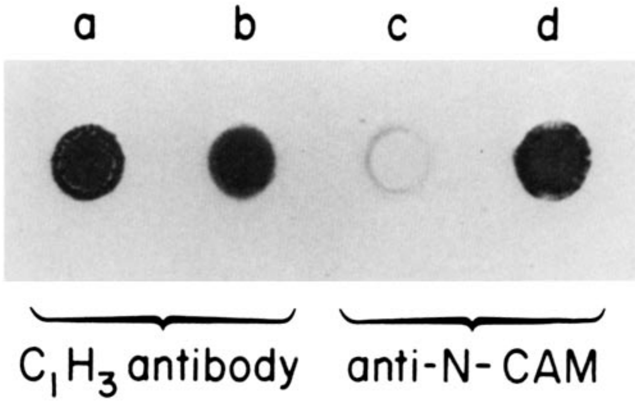


FIGURE 5 Dot-blotting of total retinal protein and retinal adheron protein using the $C_1H_3$ and anti-N-CAM monoclonal antibodies. 10 $\mu$g of total retinal protein (*b* and *d*) or 25 $\mu$g of retinal adheron protein (*a* and *c*) were blotted onto nitrocellulose. The filter was then incubated with $C_1H_3$ monoclonal antibody and HRP-conjugated goat anti-rat IgG or anti-N-CAM monoclonal antibody and HRP-conjugated goat anti-mouse IgG. Binding was visualized by reaction with 3,3′-diaminobenzidine and 0.01% hydrogen peroxide. The $C_1H_3$ monoclonal antibody binds to protein in the adheron preparation, while anti-N-CAM monoclonal antibody shows no reactivity with the adheron.

As described previously, it has been demonstrated that the $C_1H_3$ monoclonal antibody also recognizes a 140,000-mol-wt polypeptide that is distinct from the 170,000-mol-wt protein and that is only detected in early retinal tissue (12). Because it appears that the antigenic determinant participates in cell-adheron binding, and that this determinant is shared by the 170,000-mol-wt protein, we examined whether immunopurified 140,000-mol-wt $C_1H_3$ polypeptide could impair cell-substratum adhesion. To immunopurify only the 140,000-mol-wt antigen, we solubilized 1,000 embryonic day-7 retinas (the 170,000-mol-wt molecule is not present at detectable levels in the retina at this age [12]) in NP-40, and chromatographed twice on a $C_1H_3$ monoclonal antibody affinity column. PAGE of an aliquot of the 400 $\mu$g of protein that was eluted from the column with diethylamine indicated that the antigen was free of 170,000-mol-wt antigen (data not shown). Incubation of day 12 retinal cells with 10 $\mu$g of this material resulted in a pronounced inhibition of cell-substratum adhesion (Fig. 7). Collectively, these data have shown that both $C_1H_3$ antigens contain an antigenic determinant that is capable of binding to retinal cells and blocking cell to adheron binding.

## *Can Surfaces Coated with Immunopurified 170,000-mol-wt $C_1H_3$ Polypeptide Promote Cell-Substratum Adhesion?*

To demonstrate directly that the 170,000-mol-wt $C_1H_3$ protein mediates cell to adheron binding, we derivatized glass scintillation vials with $\gamma$-aminopropyl-triethoxysilane, and subsequently coated them with purified 170,000-mol-wt protein. Vials coated with partially purified protein or total adheron protein were capable of promoting cell-substratum adhesion (Fig. 8). The specificity of the response is demonstrated by the failure of the 100,000 *g* supernatant of conditioned medium to stimulate cell attachment. This result indicated that the 170,000-mol-wt $C_1H_3$ protein by itself can promote retinal cell-substratum adhesion. To rule out any possibility that the partially purified antigen promotes adhesion as a result of the presence of $C_1H_3$ monoclonal antibody in the preparation (with the antibody arising due to its release from the immunoaffinity column during elution), we assessed the ability of vials coated with small quantities of $C_1H_3$ ascites fluid to promote adhesion. Amounts of $C_1H_3$ ascites protein equivalent to the total of 170,000-mol-wt protein used to coat the dish did not support the attachment of retinal cells to the substratum (Fig. 8). Note, however, that significantly higher levels of the 170,000-mol-wt polypeptide were required to coat the dish to promote adhesion than were required when the protein was present in the form of adherons. Possible explanations for this apparent discrepancy will be presented in the Discussion.

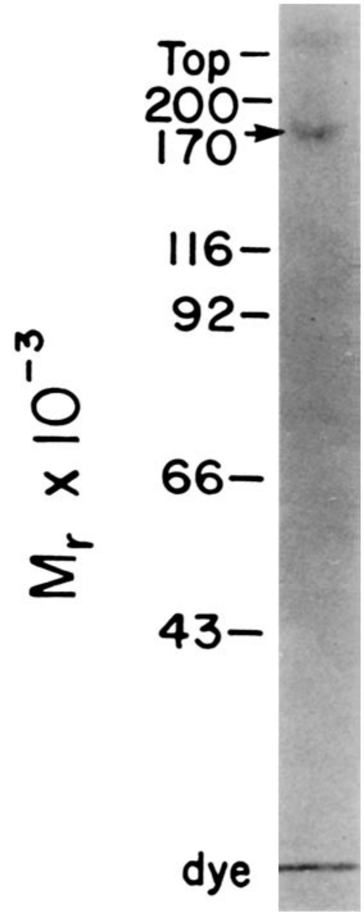


FIGURE 6 SDS PAGE of immunopurified 170,000-mol-wt $C_1H_3$ polypeptide. Membranes from 80 embryonic day-14 chick brains were solubilized in buffer A containing 1 mM EDTA, 0.5% NP-40 (pH 8.2) and incubated by end-over-end shaking with 3 ml of $C_1H_3$ monoclonal antibody-Sepharose 4B. The Sepharose was collected in a column and eluted with three column vol of solubilization buffer. Bound protein was eluted with 2.5 column vol of buffer A containing 1 mM EDTA, 0.5% NP-40, 50 mM diethylamine (pH 11.5). Detergent was removed as described in Materials and Methods, and aliquots of immunopurified protein were then heated to 100°C for 5 min in electrophoresis sample buffer and separated on a 7.5% polyacrylamide gel. Coomassie Blue staining of 15 $\mu$g of immunopurified protein, separated by electrophoresis as described, is shown. The band at 43,000-mol-wt probably represents actin, since it is not detected by immunoblotting with the $C_1H_3$ antibody (data not shown).

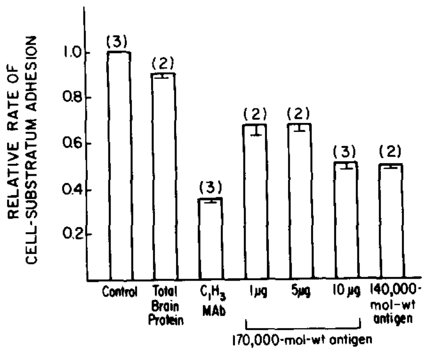


FIGURE 7 Inhibition of attachment of labeled day 12 retinal cells to adheron-coated dishes by soluble immunopurified $C_1H_3$ antigen. Cell-substratum adhesion of metabolically labeled day 12 retinal cells was measured, as previously described, in the presence of immunopurified $C_1H_3$ antigen. Retinal cells were incubated 30 min at room temperature with 1–10 μg of 170,000-mol-wt protein, or 10 μg of 140,000-mol-wt protein, washed three times with EBSS that contained 0.2% BSA, and added to adheron-coated dishes. As controls, retinal cells were either incubated with DME–15% horse serum or 10 μg of total brain protein that had been processed as described for the purification of the $C_1H_3$ antigen. Number of experiments conducted (duplicate assays per experiment) are indicated in parentheses.

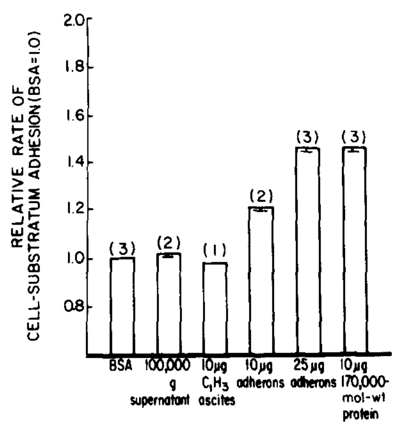


FIGURE 8 Attachment of dissociated retinal cells to derivatized glass vials. Standard glass scintillation vials were derivatized with γ-aminopropyl-triethoxysilane as described under Materials and Methods. The vials were then coated with (a) albumin, (b) the 100,000 g culture supernatant protein, (c) 10 or 25 μg of adheron protein, (d) 10 μg of immunopurified 170,000-mol-wt $C_1H_3$ protein, or (e) 10 μg of $C_1H_3$ ascites protein. Additional binding sites on the derivatized glass surface were blocked with EBSS that contained 0.2% albumin, and metabolically labeled day 12 retinal cells were added to the vials. After a 60-min incubation, the vials were swirled gently, the medium was aspirated, and bound cells were dissolved in Triton X-100 (13). The number of experiments conducted are indicated in parentheses.

## *Effect of Enzymatic Treatment on Cell-Substratum Adhesion*

To examine the role specific classes of macromolecules may play in adheron-mediated cell to substratum adhesion, we treated retinal cell adheron complexes with a variety of proteases and glycosaminoglycan hydrolases. After enzyme treatment, cell-substratum adhesion was measured. Preliminary experiments demonstrated that retina and brain adherons respond in an identical manner to the various enzyme treatments. Therefore, only retinal adherons were used to examine the effect of enzymatic manipulation on cell-substratum adhesion. When adheron-coated culture dishes were incubated with either chondroitinase ABC, hyaluronidase, or heparitinase (which degrades heparan sulfate), and then used to test cell-substratum adhesion, no significant effect was observed (Fig. 9). Because these enzymes failed to inhibit adhesion even when incubated overnight with adheron-coated dishes (data not shown), we suggest that the cell-binding receptor in adherons is not a glycosaminoglycan chain. The absence of any pronounced effect of these enzymes on adhesion was not likely due to their failure to break down glycosaminoglycans, since treatment of $^{35}SO_4$-labeled adherons released ~20–30% of the bound radioactivity for each enzyme tested (data not shown). These values are in close agreement with the relative proportion of the individual glycosaminoglycan species in retina adherons (11).

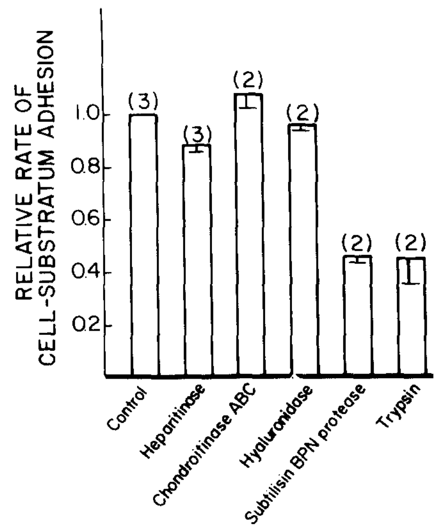


FIGURE 9 Effect of enzymatic treatments on cell-substratum adhesion. Embryonic day 12 retinal adherons were prepared as described, and used to coat 35 mm plastic Petri dishes with 30 μg of adheron protein. The adheron-coated dishes were then incubated at 37°C for 1 h with EBSS (control), 1.0 U of heparitinase, chondroitinase ABC, or hyaluronidase, or an 0.01% solution of either Subtilisin BPN protease or trypsin. The treated dishes were then washed extensively with EBSS that contained 0.2% BSA, and the rate of cell to substratum adhesion was determined after incubating the treated dishes with labeled retinal cells for 1 h. Note that the absence of any effect on adhesion by the glycosaminoglycan hydrolases was not due to their failure to degrade glycosaminoglycan. When $^{35}SO_4$-labeled adheron-coated dishes were treated with these enzymes, 30% of radioactivity was released from the dish by chondroitinase ABC, 25% by hyaluronidase, 20% by heparitinase, and 75% by Subtilisin BPN protease. These values agree with the relative proportions of these glycosaminoglycans in retinal adherons, as reported by Schubert et al. (11). The number of experiments conducted are indicated in parentheses.

In contrast to the data described above, incubation of adheron-coated dishes with several proteolytic enzymes produced a significant reduction in the ability of the adherons to bind retinal cells (Fig. 9A). It thus appears that the cell-binding molecule in adheron particles has the characteristics of a protein.

## DISCUSSION

Previous studies in our laboratory have implicated a 170,000-mol-wt neural-specific polypeptide in cell-substratum adhesion (13). This molecule is a component of adherons, which are complexes of proteins and glycosaminoglycan that promote cell to substratum adhesion of neural retinal cells (11). Although adherons provide an in vitro assay system for the analysis of neural cell-substratum adhesion, we think they possess a role in vivo as well. The 170,000-mol-wt $C_1H_3$ protein is developmentally regulated (12), with its expression in vivo corresponding to the activity of adherons in vitro. The 170,000-mol-wt protein is also neural-specific, and adheron-mediated adhesion is known to be tissue-specific, as demonstrated by the inability of muscle cells to attach significantly to retina adherons (11). The data presented has shown that brain adherons and retinal adherons, although differing in molecular composition, are essentially indistinguishable functionally, and both can bind retinal cells as well as brain cells.

We found that if the antibody was incubated with the cells or the adherons before assay, the binding of retinal cells to adherons was blocked by the $C_1H_3$ antibody. This suggests that the binding was homophilic in that molecules that contained the $C_1H_3$ determinant in retinal cells (or brain cells) and in the adherons interacted with each other. This homophilic interaction between 170,000-mol-wt protein molecules probably represents only one part of an adhesion process that may be very complex and involve additional molecules on the cell surface. The 170,000-mol-wt protein present in both cells and adherons is not solely responsible for cell to adheron binding; this is emphasized by the developmental regulation of cell to adheron binding, which is maximal at day 11–12 in the retina (11) and at day 13 in the brain, even though the concentration of 170,000-mol-wt protein continues to increase beyond that age.

The soluble 170,000-mol-wt protein has been shown to also block cell-adheron binding, which implies that it can interact with the cellular site(s) that bind to adherons, and that other components in the adheron, namely glycosaminoglycans, may not be required for binding. For example, treatment of adherons with glycosaminoglycan hydrolases does not prevent binding. However, as discussed previously, the developmental regulation of adheron activity suggests that components other than the 170,000-mol-wt $C_1H_3$ polypeptide participate in cell-adheron binding.

When used to coat glass vials, 10 $\mu$g of immunopurified 170,000-mol-wt protein promoted cell to substratum adhesion at a rate comparable to that obtained by coating the glass with 25 $\mu$g of adherons. We estimate that 25 $\mu$g of adherons contains 0.5 $\mu$g of the 170,000-mol-wt protein. Thus, the immunopurified protein is 20 times less effective than adherons in this assay, assuming that both coat the surface with equal efficiency. This discrepancy can be explained in several ways: (*a*) It is possible that the immunopurified protein is partially denatured, though immunopurification by different means has not changed the activity of the soluble protein in this assay. (*b*) The soluble, immunopurified 170,000-mol-wt protein may possess a lower affinity for retinal cells than the intact adherons. (*c*) When the 170,000-mol-wt protein is bound to the dish in the form of adherons, its local concentration is as high as it is in the adheron. The soluble protein, however, is uniformly distributed on the glass surface and much higher concentrations may therefore be required to attain the same density. Cell to cell (20) and cell to substratum adhesion (when assayed with simple carbohydrate ligands [21]) are known to require a local clustering of ligands. Although our assay systems are somewhat different, these results in related systems predict that higher concentrations of the soluble 170,000-mol-wt protein (compared with adherons) would be required to coat the glass surface in order to mediate cell adhesion effectively. This might require that a cell bind to the substratum at multiple sites in order to remain attached during the washing procedure. Recent experiments with synthetic peptides of the cell-binding domain of fibronectin have also shown that high concentrations (millimolar range) of the peptide are required to obtain 50% inhibition of cell-substratum adhesion (22). This study therefore supports our observations that large quantities of soluble antigen are needed to significantly inhibit cell to substratum adhesion.

The proposal that the 170,000-mol-wt protein in adherons binds with a higher affinity to the cellular molecule merits further analysis, particularly since a homophilic binding mechanism was suggested by our data. Homophilic binding between N-CAM molecules in neurons has been demonstrated to mediate in part neuron-neuron interactions in the nervous system (23, 24). In light of these data, cell-adheron binding would be inefficient if cellular 170,000-mol-wt proteins interacted with neighboring cellular antigens as well as they bound adheron antigens. As discussed above, the developmental regulation of adheron activity implies that additional molecules in the adheron complex are involved in cell-adheron interactions. Although the precise role of such a molecule(s) is unknown, they may promote adhesion by conferring a greater cell-binding activity to the adheron 170,000-mol-wt protein.

Experiments are currently in progress in our laboratory to further explore the possible role of additional macromolecules in adheron-mediated cell-substratum adhesion. The use of liposome vesicles that contain 170,000-mol-wt polypeptides should also show whether the vesicles bind with a higher affinity to adherons than to other vesicles. It will ultimately be of interest to determine if an active cell-adheron binding domain can be isolated, thereby permitting a more detailed characterization of the mechanism of cell-substratum attachment. Experiments are currently in progress to enzymatically digest the immunopurified 170,000-mol-wt protein, with the aim of identifying and isolating an immunoreactive fragment that can block cell to adheron binding.

Although the 170,000-mol-wt $C_1H_3$ polypeptide appears to be chemically and functionally distinct from N-CAM, its molecular weight is similar to polypeptides with an adhesive function from a host of other tissues. In embryonic nervous tissue, cell adhesion molecules similar in size to the 170,000-mol-wt $C_1H_3$ protein have been described in the neural retina (1, 18, 25) and in the cerebellum (3, 4). Cognin, a neural retina cell aggregation-promoting factor with a molecular weight of 50,000, has also been characterized (26), and is similar to the $C_1H_3$ polypeptide in that it was isolated by sedimentation from conditioned culture medium (27) and was purified from neural retina cell membranes (28). Since cognin shares characteristics with the 170,000-mol-wt $C_1H_3$ protein, it remains to be determined whether it is present in adherons and participates in cell-adheron attachment.

Macromolecules involved in cell-substratum adhesion of non-neural cell types have been identified and resemble the $C_1H_3$ antigen. For example, polypeptides with a molecular weight of 120,000–160,000 that are required for cell-matrix interactions in muscle have been described (29, 30). In fibroblast and epithelial cells, glycoproteins with a molecular weight of 120,000–140,000 that are released into the culture medium have been shown to mediate binding of cells to the substratum (31–33). In addition, the attachment of pheochromocytoma cells to culture dish plastic is enhanced by adherons from smooth muscle cell lines (34). These data suggest that cell-substratum adhesion molecules from different cell types share a number of characteristics, which include a similar molecular size and the release of the adhesion-promoting factor into the culture medium. It is thus of interest to further examine the physiological role of these molecules, particularly with respect to adhesive processes, both in vitro and in vivo.

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