A Role for Adherons in Neural Retina Cell Adhesion

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ABSTRACT Embryonic chick neural retina cells release glycoprotein complexes, termed adherons, into their culture medium. When adsorbed onto the surface of petri dishes, neural retina adherons increase the initial rate of neural retina cell adhesion; they also stimulate the rate of cell-cell aggregation. Adheron-stimulated adhesion is tissue specific, and the spontaneous aggregation of neural retina cells is inhibited by monovalent Fab' fragments prepared from an antiserum against neural retina adherons. Therefore cell surface antigenic determinants shared with adherons are involved in normal cell-cell adhesions. The particles from the heterogeneous neural retina population contain many proteins and several glycosaminoglycans. The adherons migrate as a symmetrical 12S peak on sucrose gradients and are predominantly 15-nm spheres when examined by electron microscopy. Finally, the specific activity of neural retina adherons increases from embryonic days 7 through 12 and then declines. These results suggest that glycoprotein particles may be involved in some of the adhesive interactions between neural retina cells and between the cells and their environment.

Extracellular macromolecules are involved in the adhesive interactions of many cell types. Materials released from cultured fibroblastlike and myoblast cells adhere to the surface of culture dishes and promote cell-substratum adhesion (18, 19, 26, 27). Embryonic heart cells release trypsin-sensitive molecules which associate with the culture dish and cause neurite outgrowth of ciliary ganglion cells (3). The observation that a molecule in growth-conditioned medium also promotes cell aggregation was initially made in embryonic chick neural retina cells (12, 16). Two apparently distinct molecules which are released into the culture medium have been implicated in the adhesive interactions of this cell type. The first was isolated on the basis of its ability to promote cell-cell aggregation. It was named cognin and has a molecular weight of 50,000 (16). The second molecule, isolated on the basis of its ability to neutralize antibodies against the cell surface which inhibit cell-cell aggregation, has an apparent molecular weight of 140,000 and was named nerve cell adhesion molecule (N-CAM; 31). However, during the purification of both molecules there were indications that the proteins finally isolated may initially have been associated with a higher molecular weight entity in the growth conditioned medium (16, 31).

A high molecular weight macromolecular complex which mediates cell-cell and cell-substratum adhesion has recently been isolated from the growth conditioned culture medium of muscle cells (26, 28). The skeletal muscle glycoprotein complex, termed an adheron, contains a limited number of proteins, including collagen and fibronectin, and glycosaminoglycans (GAGs). The adhesion-mediating activity migrates in sucrose gradients with a sedimentation velocity of 16S in calcium-free sucrose gradients; it aggregates in the presence of calcium ions. Similar adhesion-mediating particles have been isolated from other cell types, including smooth muscle (28) and rat nerve and glial cell lines (unpublished observation). Since the biological activity of this class of extracellular particles was similar to that of the activities thought to be involved in the aggregation of chick neural retina cells, it was asked if an adhesion-promoting activity of high molecular weight is released from cultured chick neural retina cells. The following results show that an extracellular particle containing proteins and GAGs promotes cell-substratum and cell-cell adhesion of chick neural retina cells, that the particle-mediated adhesion is cell-type specific, and that the specific activity of the adhesion-mediating complex varies with embryonic age.

MATERIALS AND METHODS

Cells and Culture: Unless otherwise indicated, neural retina tissue was separated from the pigmented epithelium of 10-d leghorn chick embryos and incubated in HEPES-buffered Dulbecco's modified Eagle's medium (DME) with 0.5% (wt/vol) crude trypsin (Difco Laboratories, Detroit, MI) for 20 min at 37°C. The cells were then rinsed three times with DME containing Spinner salts and 1% newborn calf serum, dispersed by pipetting 15 times, and placed in Spinner culture flasks containing 20 μg/ml of deoxyribonuclease I (DNase, Worthington Biochemical Co., Freehold, NJ). The cells were always incubated overnight at 100 rpm on a Bellco Glass multistir apparatus (Bellco Glass, Inc., Vineland, NJ)

THE JOURNAL OF CELL BIOLOGY · VOLUME 96 APRIL 1983 990-998

at 37°C to allow for the recovery of surface molecules damaged by the trypsin. For aggregation assays the cells were collected from a step gradient as described (2). For cell-substratum adhesion assays, the cells were removed from the Spinner cultures, washed three times with DME minus calcium plus 1% newborn calf serum, and incubated overnight in petri dishes in the same medium with 5 μCi/ml of [^{3}H]leucine.

Pigmented epithelium cells were obtained by treating the epithelium with trypsin as with neural retina, washing the cells three times with DME minus calcium plus 1% newborn calf serum, and plating the cells in petri dishes in the calcium-free medium plus 1% serum. After 24 h, the pigmented cells formed a floating epithelium, while the nonpigmented neural retina cells attached to the surface of the culture dish. The pigmented layer of cells was carefully removed and used in the experiments reported here. Heart and skeletal muscle (limb bud) cells were dissociated with 0.5% trypsin, washed, and plated directly into tissue culture dishes in DME plus 1% newborn calf serum.

Preparation of Conditioned Medium and Substrate Attached Material: Conditioned medium was prepared by washing the cells three times in serum-free DME and incubating the cells in serum-free DME for 15 h at 37°C. To prepare coated dishes, growth-conditioned medium was placed in 35-mm plastic petri dishes (Falcon Labware, Oxnard, CA) for 18 h, and the dishes were washed twice with HEPES medium. After the final wash, 2 ml of HEPES medium containing 0.2% bovine serum albumin was added.

Adhesion Assays: To assay cell-substratum adhesion, cells were labeled with [^{3}H]leucine (5 μCi/ml) in DME minus calcium plus 1% newborn calf serum for 15 h. The cells were washed three times with HEPES medium containing 0.2% albumin, and 0.2-ml aliquots were pipetted into 35-mm petri dishes to which material from growth conditioned medium had been adsorbed. At indicated times the dishes were swirled 10 times, the medium was aspirated, and the remaining attached cells were dissolved in Triton X-100 and their isotope content was determined. The data were plotted as the fraction of input cells that adhered as a function of time. Variation between duplicates was <5%. Cell-substratum adhesion-promoting activity is defined as the initial rate of adhesion per unit volume of the original culture supernatant material.

Cell aggregation was measured by the disappearance of single cells from an agitated suspension. Cells were washed twice in HEPES medium and added at 1×10^6 cells/ml to 0.5-ml aliquots of the test medium. The cells were agitated on a rotary shaker (100 rpm) at 37°C, and the disappearance of single cells was monitored with a Coulter Counter (20).

Protein and Glycosaminoglycan Assays: Cells were labeled with [^{35}S]methionine and gel electrophoresis was done in gels containing 15% acrylamide and 0.1% SDS as described (1). GAG analysis was carried out by labeling cells with [^{3}H]glucosamine or [^{35}S]sulfate, chromatographing the sample on a DEAE-cellulose column (33), and identifying the GAGs in the individual peaks by enzymatic methods (24). Streptomyces hyaluronidase (B grade, Calbiochem, San Diego, CA) was used at 50 U/ml for 24 h at 37°C. Chrondroitinase ABC and AC (Miles Laboratories, Elkhart, IN) were used at 0.5 U/ml for 24 h at 37°C. GAG breakdown was monitored by column chromatography in Sephadex G-50.

Preparation of Antiserum: Neural retina adherons were purified as described in the text. For each injection, 100 μg of particulate material in saline was mixed with an equal volume of complete Freund's adjuvant and injected subcutaneously into six spots on the backs of white New Zealand female rabbits. After four 100-μg injections equally spaced over 6 wk, the rabbits were bled and the serum was used in these experiments. IgG was purified by DEAE chromatography (6) and monovalent Fab' fragments were prepared according to Brakenbury et al. (2).

Electron Microscopy: Neural retina adherons were sprayed from a glass nebulizer at 50 μg/ml in 50% glycerol onto a mica substrate (32). The mica was then placed in a vacuum evaporator (Edwards E306) which was evacuated to 10^{-6} millibar and rotory shadowed with platinum at an angle of 5 to 10° from a twin electron beam gun. Carbon was then evaporated onto the mica to form a platinum-carbon replica. Replicas were floated onto a water surface and picked up on 500-mesh copper grids. To measure the size of the particles with greater accuracy, purified adherons were negatively stained with 0.5% uranyl formate. All grids were examined in a Hitachi HG-600 electron microscope.

RESULTS

Particulate Nature of Extracellular Adhesion Promoting Activity

Since material in growth conditioned medium of embryonic chick neural retina cells promotes homotypic cell aggregation (12), and since this type of activity resides in a high molecular weight glycoprotein complex in some other cell types (26, 28), it was asked if the adhesion-mediating material of neural retina was particulate. Two types of adhesion assays were employed. The first made use of the fact that embryonic chick neural retina cells do not adhere to plastic petric dishes, while petri dishes to which molecules from growth conditioned media are adsorbed stimulate the initial adhesion rate of retina cells. The second assay employed cell-cell aggregation. Material derived from growth conditioned media of chick neural retina cells promotes the rate of spontaneous aggregation. Since the cell-substratum assay was easier to quantitate than the homotypic aggregation assay, it was used in the majority of the experiments outlined below.

When isotopically labeled neural retina cells were plated into plastic petri dishes containing HEPES buffered culture medium and 0.2% BSA, <0.4% of the input cells adhered to the culture dish over a period of 1 h. However, when the petri dishes were exposed to serum-free growth conditioned medium from neural retina cells for 18 h, the culture dishes were extensively washed, and then the adhesion of neural retina cells was assayed, 30% of the input cells adhered after 1 h (Fig. 1*A*). To determine whether the adhesion promoting material was particulate in nature, the growth conditioned medium was centrifuged at 100,000 *g* for 3 h, and the pellet and supernatant were assayed for their ability to promote cell-substratum adhesion. Fig. 1*A* shows that approximately threefold more activity was recovered in the pellet than was originally detected in the complete growth conditioned medium, and that only a few percent of the adhesion-promoting activity remained in the supernatant after centrifugation at 100,000 *g*. By the inclusion of growth conditioned medium from cells labeled with [^{3}H]-glucosamine and [^{14}C]leucine, it was demonstrated that all of the protein and carbohydrate in the pellet adhered to the petri dish. About 5% of the protein in the growth conditioned medium and 30% of the extracellular carbohydrate was pelleted. The adhesion-mediating material was therefore a subset of the total extracellular macromolecules.

Since the adhesion-promoting activity in the 100,000 *g* pellet was greater than that in the complete growth conditioned medium, it was possible that two or more components were interacting to suppress the adhesion-promoting activity in the unfractionated growth conditioned medium. If these two activities could be separated by centrifugation, then mixing the pelleted material and the high speed supernatant to reconstitute the original growth conditioned medium should produce the original, lower, adhesion kinetics. Fig. 1*A* shows that the activity in the pellet was reduced to the predicted level when pelleted and supernatant materials were mixed. The inclusion of isotopically labeled particulate material showed that the high speed supernatant did not block the adsorption of the pelleted molecules to the substratum. These results suggest that at least two activities in conditioned medium are involved in neural retina adhesion.

Some cell-substratum adhesions can be inhibited by exogenous GAGs, which apparently act as haptens to block protein-GAG or GAG-GAG interactions between the cell surface and substratum-bound adhesion-promoting molecules (26, 28). Most of the cell lines tested have a characteristic inhibition profile with respect to the type and amount of GAGs which inhibit cell-substratum adhesion (see, for example, reference 28). When the ability of exogenous GAGs to inhibit cell-substratum adhesion of neural retina cells to substrata prepared from total growth conditioned medium, the high

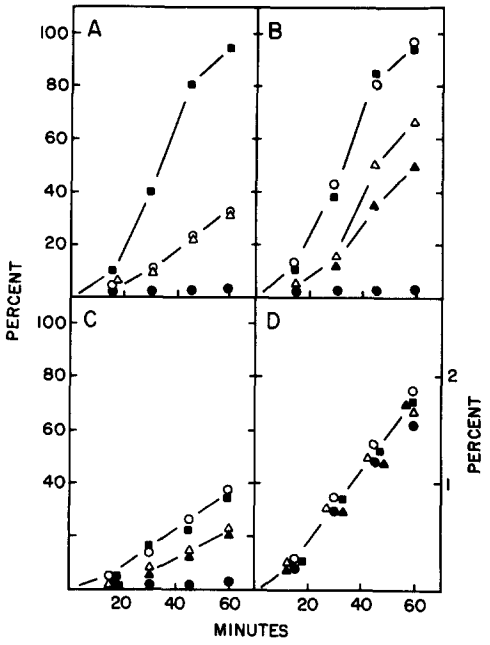

FIGURE 1 Distribution and GAG specificity of adhesion mediating material. Serum-free growth conditioned medium was prepared from 10-d chick neural retina cultures. The conditioned medium was centrifuged for 5 min at 500 *g* to remove cells and at 20,000 *g* for 15 min to remove cell debris. These two initial centrifugations were done in all of the following experiments. Half of the medium was placed on ice, and the other half centrifuged at 100,000 *g* for 3 h, and separated into pellet and supernatant. The pellet was washed once with serum-free medium and resuspended to the original (same) volume as the medium and high speed supernatant. Equal amounts of each were incubated overnight in plastic petri dishes. In one case the pellet and high speed supernatant were recombined prior to the incubation. The rate of adhesion of isotopically labeled 10-d chick neural retina cells to surfaces coated with the above three fractions was also determined in the presence or absence of various glycosaminoglycans at 10^{-3} M with respect to glucuronic acid, assuming it to be a free molecule. The percent of input cells (inferred from the fraction of original isotope in the cells) adhering to the dishes was plotted as a function of time. Less than 0.4% of the input cells adhered to petri dishes alone after 60 min (data not presented). The error between duplicate samples was <5%. (*A*) Adhesion of neural retinal cells to: (△) material adsorbed from total conditioned medium; (●) supernatant of growth conditioned medium after centrifugation at 100,000 *g*. (■) adhesion to 100,000-*g* pellet; (○) recombined pellet and 100,000-*g* supernatant. (*B*) GAG inhibition of adhesion to material adsorbed from 100,000-*g* pellet: (■) pelleted material alone; (○) pelleted material plus hyaluronic acid; (△) chondroitin-4-sulfate; (▲) dermatan sulfate (chondroitin-6-sulfate was indistinguishable from dermatan sulfate); (●) heparan sulfate and heparin, maximum adhesion was 5% at 60 min. (*C*) GAG inhibition of adhesion to material adsorbed from total growth conditioned medium: (■) total growth conditioned medium; remaining symbols same as in *B*. Left ordinate. (*D*) GAG inhibition of adhesion to material adsorbed from the 100,000-*g* supernatant: (■) 100,000-*g* supernatant; remaining symbols same as in *B*. The effects of heparin and heparan sulfate were indistinguishable. Right ordinate.

speed pellet, and the 100,000-*g* supernatant were tested, heparan sulfate and heparin completely blocked the adhesion of neural retina cells to total and particulate material but poorly inhibited the binding of cells to the high speed supernatant material (Fig. 1 *B*, *C*, and *D*). Similarly, the chondroitin sulfates partially blocked adhesion of cells to the pellet and total supernatant material but were ineffective with respect to the surface coated with high speed supernatant molecules. Hyaluronic acid did not alter adhesion to any fraction. These data suggest that the small amount of activity remaining in the soluble pool of growth conditioned medium after the 3-h centrifugation is distinct from that which is pelleted. Since this residual activity represents <2% of the total, its characterization will not be discussed further here. The nature of the adhesion inhibitor activity in the high speed supernatant is currently under investigation.

To determine whether the initial rate of adhesion of neural retina cells to substrata coated with particulate material was proportional to the amount of protein adsorbed to the dish, neural retina cells were allowed to adhere to dishes coated with different concentrations of particulate material. Isotopically labeled samples showed that all of the input protein at each concentration was adsorbed to the culture dish. Fig. 2 indicates that there was a linear relationship between the amount of protein on the substratum and the fraction of input cells adhered at 30 min. Half-maximal adhesion at 30 min was caused by about 40 μg of protein per 35-mm culture dish.

The assay most widely employed to quantitate cellular adhesion in the neural retina system has been aggregation (12, 16, 31). Two varieties of aggregation assays were used. The first determines an increase in aggregate size (12) and the second makes use of the fact that molecules involved in aggregation neutralize the adhesion-inhibiting effect of antisera prepared against the whole cell surface (11, 31). Attempts were made to examine the effect of the particulate fraction of growth conditioned medium on the aggregate size of spontaneously aggregating neural retina cultures. No statistically significant difference was observed. However, when the rate of disappearance of single cells was assayed, the cell suspensions containing the neural retina materials pelleted by centrifugation at 100,000 *g* aggregated at a greater rate than those in the absence of this material (Fig 3 *B*). Both the spontaneous and particle-induced aggregation were calcium independent, for they occurred at the same rate in calcium- and magnesium-free medium plus 5 ×

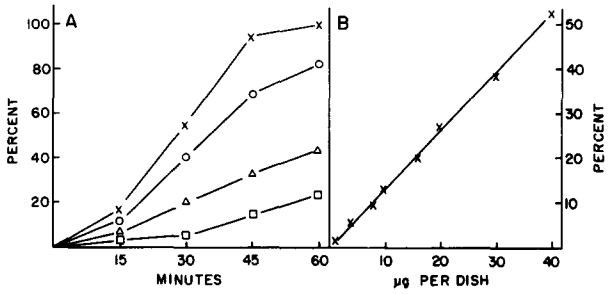

FIGURE 2 Dose-response curve for cell-substratum adhesion. Particulate material from growth conditioned medium was prepared from 10-d-old chick neural retina as described in Fig. 1 and incubated overnight at 37°C at various concentrations in 35-mm petri dishes. Approximately 5 μg of particulate material was released into the medium per retina per 15 h. All of the added carbohydrate and protein adhered to the culture dish as determined by the inclusion of isotopically labeled material in some samples. The adhesion of 11 d neural retina cells to the different substrate was then assayed as described in Fig. 1. *A* shows the percent of input cells adhered to dishes treated with different amounts of material. Smooth curves were drawn through the binding curves and the percent of input cells adhered at 30 min then plotted as a function of particle protein on the culture dish (*B*). (*A*) (□) 4 μg protein per dish; (△) 16 μg; (○) 30 μg; (X) 50 μg. (*B*) Relationship between the amount of protein bound and adhesion rate.

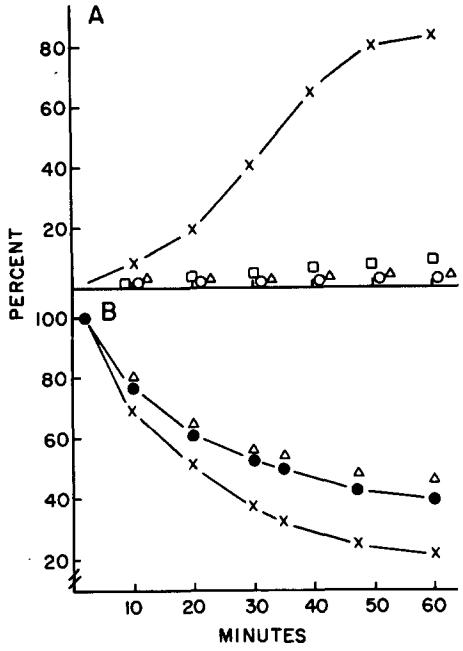

FIGURE 3 Stimulation of cell-substratum adhesion and cell aggregation by particulate material from growth conditioned medium. Chick neural retina, pigmented epithelium, or skeletal muscle cells were removed from the embryo on day 10, dissociated with trypsin, incubated for 24 h in Spinner culture, washed three times, and then incubated for an additional 18 h in serum-free DME medium. As in all of the experiments employing conditioned medium, medium was then centrifuged for 5 min at 500 *g* and for 15 min at 20,000 *g*. The supernatant was centrifuged at 100,000 *g* for 3 h. The pellet was resuspended in HEPES medium and either incubated in 35-mm petri dishes at 30 μg protein per dish overnight at 37°C or used in the aggregation assay at 30 μg protein/ml. The adhesion of 11-d-old embryonic neural retinal cells to coated substrata was assayed as described in Fig. 1. To assay aggregation, 11-d neural retina cells were dissociated with trypsin, incubated in Spinner's medium overnight, and their agglutination at 2×10^6 cells/ml was monitored by measuring the disappearance of single cells by a Coulter Counter. (*A*) Cell-substratum adhesion. Percent of input neural retina cells attached to substratum plotted as a function of time: (○) petri dishes alone; (X) neural retina-coated substratum; (△) pigmented epithelium-coated substratum; (□) skeletal muscle cell-coated substratum. (*B*) Aggregation of neural retina cells: (●) control, no additive; (X) neural retina particulate material; (△) skeletal muscle cell particulate material. Pigmented epithelium was also ineffective (data not shown).

10^{-4} M EGTA. Although this aggregation assay was not very sensitive, it did suggest that the particulate material may be involved in cell-cell aggregation. Further evidence for this will be presented later.

Cell-Type Specificity of Adhesions

The above data suggest that the particulate material in growth conditioned medium may mediate some cell-substratum and cell-cell adhesions. To determine the cell type specificity of these interactions, particulate material was prepared from pigmented epithelium, neural retina, and skeletal muscle myoblasts from cells of 10-d chick embryos. The material was adsorbed to petri dishes at 30 μg/dish, and the ability of neural retina cells to adhere to these different substrata determined. Fig. 3*A* shows that neural retina cells adhered only to the neural retina-derived substratum. Similarly, material from embryonic skeletal muscle did not enhance the rate of cell-cell aggregation (Fig. 3 *B*). It follows that there is at least a limited amount of tissue specificity in the ability of the particulate fractions of growth conditioned media to mediate cellular adhesion.

Size and Composition of the Adhesion Mediating Material

When the growth conditioned medium of neural retina cells was centrifuged for 3 h at 100,000 *g*, most of the adhesion- and aggregation-promoting activities were found in the pellet. If the adhesion-promoting material is a defined molecule or macromolecular complex, it should sediment in a sucrose gradient and the activity mediating adhesion should be associated with this entity. Since GAGs may be involved in the adhesion of neural retina cells (Fig. 1), cells were incubated for 18 h in serum-free medium with [^{35}S]sulfate, [^{3}H]glucosamine, or [^{35}S]methionine; unlabeled serum-free conditioned media were also prepared. The conditioned media were centrifuged at 100,000 *g* for 3 h, the supernatant was aspirated, and the pellet was carefully washed twice with HEPES medium and resuspended in 0.01 M HEPES buffer, pH 7.1. The samples were then sedimented in 5–20% (wt/vol) sucrose gradients in the same buffer for 20 h. The $^{35}SO_4$ label defined a peak at 12S that sedimented about one-third of the way into the gradient (Fig. 4). This particle has been termed an adheron (28). When the ability of each gradient fraction of the unlabeled material to mediate cell-substratum adhesion and cell aggregation was assayed, an increase in adhesion and aggregation of neural retina cells was effected only by the gradient fractions that coincided with the presence of the $^{35}SO_4$ isotope. Since the adherons from skeletal muscle sediment as a symmetrical 16S peak in the absence of calcium but aggregate and pellet in the presence of exogenous calcium (26), it was asked if calcium altered the mobility of the neural retina particle. Concentrations of calcium up to 10 mM had no effect on particle sedimentation.

These data do, however, suggest that the 12S particles are aggregated in the growth conditioned medium, for a 12S particle would not be quantitatively pelleted by centrifugation at 100,000 *g* for 3 h. A 10-h centrifugation would be required under the conditions used in these experiments. Once the particles are removed from growth conditioned medium and resuspended in buffer, they must dissociate to form the 12S adheron.

The above experiment suggests that particles contained both protein and GAGs. Therefore, cells were labeled with [^{35}S]-methionine, [^{3}H]glucosamine, or $^{35}SO_4$, the culture medium was centrifuged at 100,000 *g* for 3 h, and the pellets were sedimented on sucrose gradients. The isotopically labeled 12S peaks were collected, assayed for protein on polyacrylamide gels, and the GAG content was determined by column chromatography and enzymatic analysis. When particles labeled with ^{35}S-methionine were electrophoresed on acrylamide gels, several major bands were observed alone with many bands of lesser intensity (Fig. 5, lane *3*). Fig. 5 also shows the proteins in total growth conditioned medium (Fig. 5, lane *1*) and medium after centrifugation at 100,000 *g* for 3 h (Fig. 5, lane *2*). The major bands in the particle are a 20,000-mol wt protein (Fig. 5, *D*) a 43,000-mol wt protein (*C*), and a group of closely migrating proteins near 50,000 mol wt (*B*) (Fig. 6). A protein of about 140,000 mol wt (Fig. 6, *A*) was also present. A "smear" of isotope at the top of the gels is largely removed from the growth conditioned medium by high speed centrifugation.

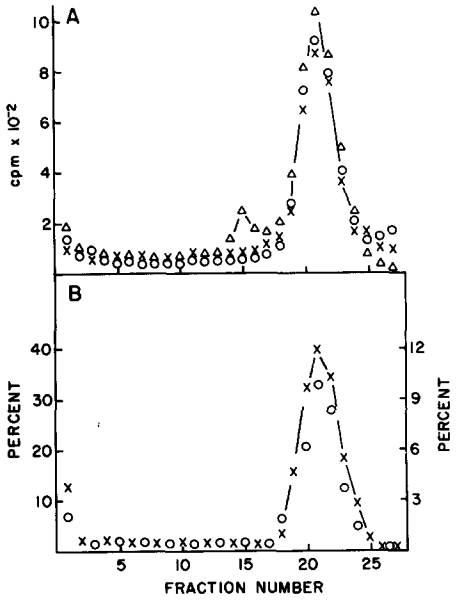

FIGURE 4 Sucrose gradients of chick neural retina adherons. Cells were dissociated from 10-d neural retina and incubated overnight in Spinner culture. The cells were then washed three times and isotopically labeled with $^{35}SO_4$, [^{35}S]methionine, or [^{3}H]glucosamine for 18 h in serum-free medium; another group of cells was incubated without isotope. The conditioned media were then centrifuged as described in Fig. 1 and the 100,000-*g* pellets centrifuged into 5% to 20% sucrose gradients in 0.01 M HEPES, pH 7.1. Centrifugation was done for 20 h at 4°C in a SW41 Beckman rotor at 36,000 rpm. The top of the gradient is at the right of the graph. The recovery of input isotope in the gradient was >92% for all three isotopes. [^{3}H]Glucosamine was co-sedimented with [^{35}S]methionine and [^{35}S]sulfate in separate experiments. Each gradient fraction containing neural retina adheron was divided into two aliquots. One was diluted into HEPES medium to determine its effect on neural retina aggregation as described in Fig. 3. The data are expressed as the percent difference in aggregation between experimental and control cells after 40-min incubation. The other was diluted into HEPES medium and incubated in petri dishes overnight at 37°C. The following day the adhesion of isotopically labeled neural retina cells was determined at 40-min incubation as in Fig. 1. (*A*) Isotopically labeled adherons: (○) $^{35}SO_4$; (△) [^{35}S]methionine; (X) [^{3}H]glucosamine. (*B*) Adhesion and aggregation of cells by gradient fractions: (X) percent of input cells adhered at 40 min (left ordinate); (○) percent difference in aggregation (right ordinate).

None of these bands has yet been characterized.

To determine the nature of the GAGs associated with the 12S particle, the $^{35}SO_4$ and [^{3}H]glucosamine-labeled 12S peak from sucrose gradients was digested with Pronase and chromatographed on a DEAE-cellulose column to separate the classes of GAGs (Fig. 6 and Table I). Each GAG was identified by its mobility on the column in comparison to that of known GAGs and by its susceptibility to being degraded by enzymes of known specificity. Peak *A* (Fig. 6) contained a very small amount of sulfated material and was not degraded by fungal or testicular hyaluronidase, chondroitinase ABC, or by nitrous acid. Its identity is unknown. Peak *B* (Fig. 6) is hyaluronic acid because it contained no sulfate, was degraded by fungal hyaluronidase, and was not degraded by nitrous acid. Peak *C* (Fig. 6) was degraded by nitrous acid and it contained sulfate, suggesting that it is heparan sulfate. Peak *D* (Fig. 6) contained sulfate and was degraded by chrondoitinase ABC but not by fungal hyaluronidase nor by nitrous acid and thus is chon-

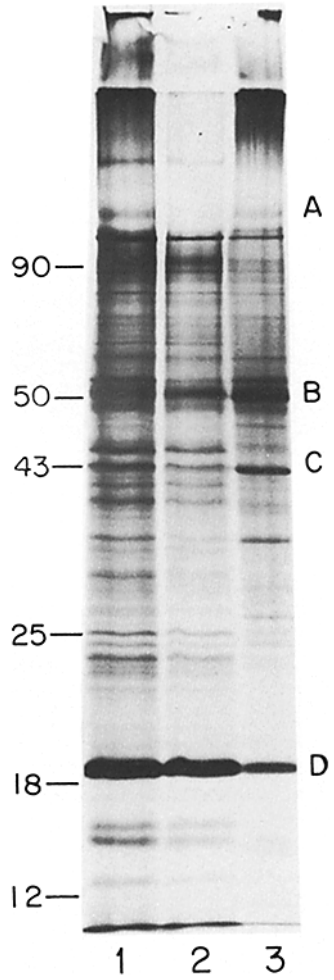

FIGURE 5 SDS acrylamide gels of adheron proteins from chick neural retina. Cells from 10-d-old embryos were dissociated with trypsin, placed in Spinner culture overnight, washed and then labeled with [^{35}S]methionine in serum-free medium for 18 h. The 12S particles were isolated on a sucrose gradient as described in Fig. 4, and the proteins electrophoresed on 15% polyacrylamide gels containing 0.1% SDS along with the isotopically labeled growth conditioned medium before and after the 100,000 *g* centrifugation used to initially isolate the particle. 120,000 cpm were applied to lanes *1* and *2*, and 30,000 to lane *3*. Lane *3* was exposed approximately four times longer than the others. Lane *1*: Total growth conditioned medium. Lane *2*: Medium after high speed centrifugation. Lane *3*: Particle purified on sucrose gradient. The total particulate material and that purified on sucrose gradients were indistinguishable by this assay. Molecular weight measurements, $\times 10^{-3}$.

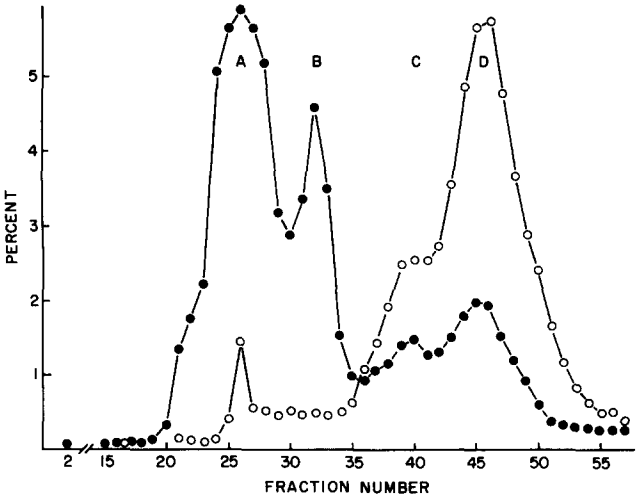

FIGURE 6 Elution profile of GAGs from an ion exchange column. Neural retina cells were labeled with [^{3}H]glucosamine and $^{35}SO_4$ for 18 h and the culture medium supernatant was centrifuged (100,000 *g* for 3 h). The pellet was applied to a 5–20% sucrose gradient, the peak isolated, digested with pronase, and the GAGs were run on a 1 × 20 cm DE-52 column (DE-52, Whatman, England). The starting buffer was 0.002 M Tris, pH 7.5, 0.01 M NaCl, and the GAGS were eluted with a linear gradient between 0.01 and 1.0 M NaCl in 0.002 M Tris, pH 7.5, followed by a wash with 5 M NaCl. The data are expressed as the percentage of the total recovered counts in each fraction. (○) [^{35}S]sulfate label; (●) [^{3}H]glucosamine label.

TABLE I

GAG Composition of Chick Neural Retina Fractions

GAG Peak	Cells	Medium	Adheron
Unknown* (A)‡	56	38	51
Hyaluronic acid (B)	16	25	17
Heparan sulfate (C)	18	18	11
Chondroitin sulfate (D)	9	18	20

GAGs were isolated from the cells, total growth conditioned medium, and 12S adheron of 11-d neural retina cells isotopically labeled with $^{35}SO_4$ and ^{3}H glucosamine. The cells were removed from the culture dish with a rubber policeman, pelleted, lysed by freeze-thawing, and digested with pronase in the presence of DNAase (10 μg/ml) as with the conditioned medium and adherons. GAGs were identified by column chromatography and enzymatic digestion, and labeled A–D corresponding to Fig. 6. 10% of the total GAGs synthesized during the 18-h labeling period were found in the culture medium, and of the extracellular material 30% or ~3% of the total incorporated isotope, was in the particles. Each column indicates the percentage of total [^{3}H]glucosamine found in the individual carbohydrates represented by each peak (A–D, Fig. 6) of the elution profile of GAGs from the DEAE column.

* Not degradable by fungal or testicular hyaluronidase, chondroitinase ABC, or nitrous acid.

‡ See Fig. 6.

droitin sulfate. The relative distribution of the various classes of GAGs in cells, conditioned medium, and particle is also shown in Table I.

Ultrastructure of Neural Retina Adheron

Since the neural retina adheron sedimented as a single peak on sucrose gradients, it was likely that the particle was relatively homogeneous in size. To obtain a better estimate of the size and homogeneity the adherons, the purified particles were examined by electron microscopy using both negative staining and rotory shadowing. Fig. 7*A* and *B* show that the particles were spherical, although irregular shapes were also observed. Occasionally, the particles had a star-burst or pinwheel configuration (Fig. 7*C*). A histogram of the adheron diameters is presented in Fig. 8. Approximately 60% of the particles had diameters of 15 to 18 nm, with a mean diameter of 15.4 ± 2.0 nm.

The Effect of Monovalent Antisera on Aggregation and Adhesion

To further characterize the nature of the molecules and their interactions which lead to adheron-stimulated cellular adhesion, it is advantageous to have an antiserum which recognizes the structures involved in the adhesion process. Therefore antisera were prepared in rabbits against the purified neural retina adheron. Of the two rabbits injected, one produced antibodies which quantitatively precipitated radioactive neural retina adherons and inhibited cell-substratum and cell-cell adhesion. Fig. 9*A* shows that monovalent Fab′ fragments inhibited the spontaneous aggregation of neural retina cells but had no effect on the aggregation of chick skeletal muscle myoblasts. This antiserum also inhibited cellular adhesion of neural retina cells to substrata coated with neural retina adherons. However, the inclusion of either preimmune IgG or Fab′ protein in the adhesion assay also completely inhibited adhesion of the cells to the particle-coated surface, probably by binding to the surface of the culture dish and nonspecifically inhibiting adhesion (see reference 25). It was therefore necessary to preincubate the cells at 4°C with the Fab′ protein and wash the cells once before their adhesive properties were assayed. When this was done, cell-substratum adhesion of neural retina cells was inhibited, while the adhesion of skeletal

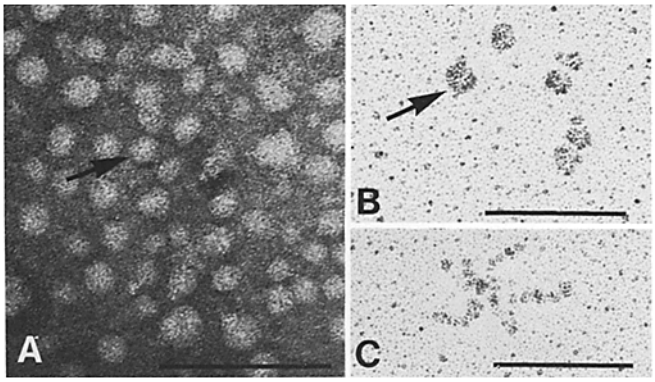

FIGURE 7 Electron micrograph of adherons. Adherons were either negatively stained with uranyl formate (*A*) or sprayed and rotory shadowed with platinum (*B* and *C*). In some preparations the particles appear to generate pinwheels (*C*). Arrows indicate individual adherons. Bars, 100 nm.

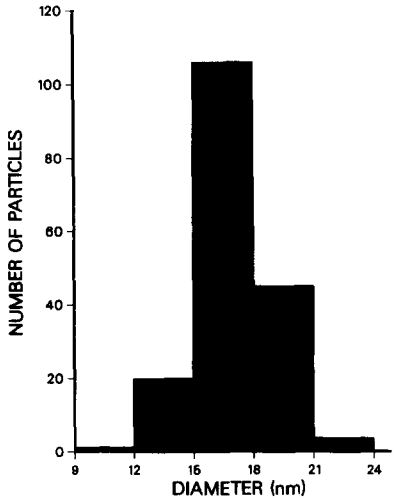

FIGURE 8 Histogram of adheron diameters. Maximum particle diameters were measured from micrographs of negatively stained preparations and grouped in 3 nm size classes.

muscle myoblast cells to myoblast particles was not altered (Fig. 9*B*). Neither cell-cell nor cell-substratum adhesions of neural retina cells were, however, completely blocked by 1 mg/ml of Fab′, suggesting that the titer of the antiserum against some of the critical determinants required in the adhesion processes was low.

The above results suggest that there are molecules on the surface of cells which share antigenic determinants with the 12S neural retina particle and which are involved in cell-cell and cell-substratum adhesion. If this argument is true, then purified adherons should block the inhibitory effect of Fab′ fragments on spontaneous neural retina adhesion (2, 11). When increasing amounts of neural retina adherons were incubated with Fab′ fragments and then the effectiveness of the Fab′ in blocking spontaneous aggregation was tested, the neural retina adherons were able to neutralize the blocking activity of the antibody (Fig. 10). Those of skeletal muscle were inactive. On the basis of these antibody experiments and the ability of neural retina adherons to increase the rate of cell aggregation (Fig. 3), it can be concluded that one or more components of the neural retina adheron are involved in cell aggregation.

Developmental Regulation of Adhesion Mediating Activity

Previous investigations have suggested that the amount of cell aggregation-promoting activity in chick neural retina varies

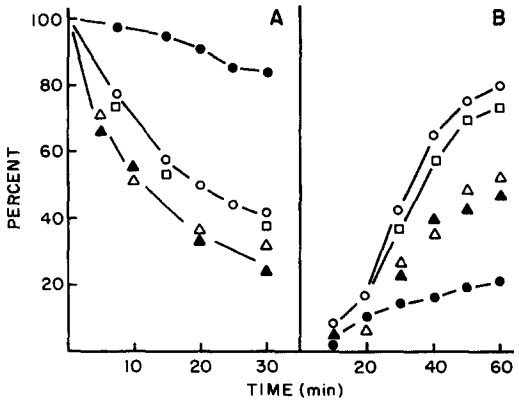

FIGURE 9 Effect of monovalent antibody on cell aggregation and cell-substratum adhesion. 10-d embryonic chick neural retina cells were dissociated with trypsin and incubated overnight in Spinner culture for the aggregation assays or in DME minus calcium plus [^{3}H]leucine for the cell-substratum adhesion assays. Skeletal muscle myoblasts were dissociated with 5×10^{-4} M EGTA immediately before the adhesion assays. The aggregation assay was done as described in Fig. 3 except that cells were incubated with 1 mg/ml of antiadheron or pre-bleed Fab' at 4°C for 20 min, the cells warmed to 37°C in a water bath, and then the disappearance of single cells from the agitated suspension monitored. For the cell-substratum adhesion assays, the kinetics of adhesion of isotopically labeled cells to substrata containing 30 μg/dish of purified skeletal muscle or neural retina adherons was determined as described in Fig. 1. To assay the effect of antiserum on adhesion, 2×10^6 cells were incubated with 1 mg/ml of Fab' at 4°C for 30 min, washed once with cold HEPES medium plus 0.2% BSA, and then used in the adhesion assay. (*A*) Cell aggregation, plotted as percent decrease in single cells as a function of time: (○) Neural retina cells, spontaneous aggregation; (●) neural retina cells, antiadheron Fab'; (□) neural retina cells, pre-bleed Fab'; (△) myoblast cells, spontaneous aggregation; (▲) myoblast cells, antineural retina adheron Fab'. (*B*) Cell-substratum adhesion, plotted as percent of input cells which adhered to the substratum: (○) Neural retina cells alone to neural retina particles; (□) neural retina cells, pre-bleed Fab'; (●) neural retina cells, antiadheron Fab'; (△) myoblast cells alone to myoblast particles; (▲) myoblast cells, anti-neural retina adheron Fab'.

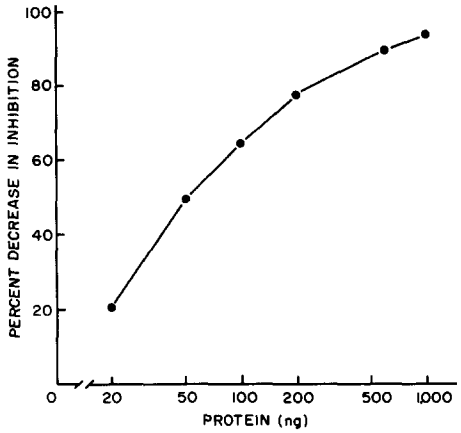

FIGURE 10 The neutralization of antiadheron Fab' inhibition of spontaneous aggregation by neural retina adherons. 1 mg of anti-adheron Fab' was incubated with varying amounts of neural retina adheron in 0.5 ml of HEPES medium containing 30 μg/ml of DNAase at 22°C for 30 min. The tubes were then cooled to 4°C and 1×10^6 dissociated cold cells were added and the incubation continued at 4°C for another 20 min. The tubes were then mixed vigorously, placed in a 37°C water bath for two minutes while manually shaking, and then put on a rotary shaker at 37°C, and the disappearance of single cells monitored as described in Fig. 3. The data are presented as the percent decrease in antiadheron inhibition at 30 min plotted as a function of adheron protein.

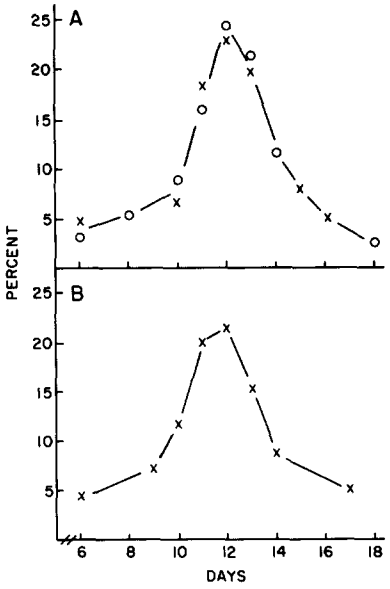

FIGURE 11 Developmental time course of neural retina adhesion promoting activities. Cells and adherons from neural retina tissue of various embryonic ages were prepared as described in Fig. 1. The age of the particle or cells was that of the embryonic age from which the cells were removed plus the time the cells were in culture. For example, if particles were prepared from 10-d embryo cells the cells were dissociated and incubated overnight in Spinner culture and again overnight in serum-free medium to prepare the adherons, the adherons were designated day 12. If cells from 10-d-old embryos were dissociated and isotopically labeled overnight, they were designated day-11 cells. The data are represented as the percent of input cells which adhere at 30 min to the dishes coated with 10 μg of adherons from the designated embryonic day. (*A*) Adhesion of 11-d neural retina cells to particle-coated substrata from various embryonic days (abscissa). X's and O's are data from two separate experiments. (*B*) Adhesion of cells from various embryonic days (abscissa) to surfaces coated with particles from day-12 cells and to new petri dishes (X) particle coated dishes.

with embryonic age (8, 22). To determine whether there is a similar change with development in the cell-substratum adhesion-promoting activity described here, particles were isolated from growth conditioned media of neural retina cells of embryonic ages 6–17 d. The apparent size of the particle remained constant on sucrose gradients. The specific activity of the adhesion-promoting activity was determined by assaying the extent of adhesion of test cells from embryonic day 12 to substrata derived from days 6 through 17 (Fig. 11*A*) or by using substrata exclusively prepared from day 11 conditioned media and varying the embryonic age of the test cells (Fig. 11*B*). These data show that the specific activity (adhesion-promoting activity per unit protein) using the two fixed parameters increases approximately fourfold between embryonic day 10 and 12, and subsequently declines. This change is contemporaneous with major developmental changes in the retina, for the overall histogenesis of the chick retina is complete at about embryonic day 18.

DISCUSSION

The following conclusions may be made from the above data: (*a*) Cultured embryonic chick neural retina cells release a particle into the growth medium which stimulates cell-substratum

adhesion and which increases the rate of cell-cell aggregation (Figs. 1–3). (*b*) This particulate activity is cell-type specific (Figs. 3 and 9). (*c*) The adhesion-stimulating activity is contained in a glycoprotein complex which sediments at 12S on sucrose gradients and is composed of a large number of proteins and several GAGs (Figs. 4, 5, and 6). (*d*) Electron microscopy indicates that neural retina adherons are spheres ~15 nm in diameter (Figs. 7 and 8). (*e*) One or more of the antigenic determinants on the 12S particle mediates spontaneous cell-cell aggregation, for Fab′ fragments of an antiserum against adherons inhibit cell aggregation, and purified adherons neutralize the adhesion-blocking effect of the monovalent antibody (Figs. 9 and 10). (*f*) The specific activity of the particle varies during embryonic development (Fig. 11).

Although a number of activities involved in the adhesive interactions between chick neural retina cells have been described (12, 14, 23, 30, 31), only three macromolecules have been purified to homogeneity and shown to be involved in the adhesion process. The first was a 50,000-mol wt protein (cognin) isolated on the basis of its ability to promote the aggregation of neural retina cells (16). In contrast, ligatin, a protein of 10,000 mol wt, inhibits the cell-cell adhesive interactions of neural retina cells (15, 17). The third protein, designated nerve cell adhesion molecule (N-CAM), was isolated on its ability to neutralize an anti-neural retina serum's ability to inhibit the spontaneous aggregation of neural retina cells (31). There is, however, no published evidence showing that this 140,000-mol wt protein can directly stimulate or inhibit neural retina cell adhesion. All three proteins are associated with the plasma membrane, and both cognin and N-CAM were isolated from growth conditioned medium. During the initial purification of both N-CAM and cognin, data were presented suggesting that they existed in higher molecular weight forms. N-CAM had an apparent molecular weight of ~400,00 on a sizing column (31), and cognin was pelleted by high speed centrifugation (16). Fig. 5 shows that the adherons contain several proteins ~50,000 mol wt, and lesser amounts of a 140,000-mol wt protein. It is therefore possible that both previously described proteins are involved in the adheron-mediated adhesion process. Moreover, since the 50,000-mol wt cognin protein alone is capable of aggregating neural retina cells (16), the complete particle may not be necessary for aggregating cells. The role of individual proteins within the neural retina adherons and their relationship to published activities are under investigation.

The chick neural retina adherons are composed of both glycosaminoglycans (GAGs), an unknown polysaccharide, and a large number of proteins. Hyaluronic acid and chondroitin sulfate are the major GAGs, while ~50% of the total glucosamine in the particle is in an unsulfated polysaccharide that is not susceptible to degradation by hyaluronidase, chondroitinase, or nitrous acid. Its chemistry is currently being studied. The number of proteins in the chick neural retina adherons is several-fold greater than that of the clonal L6 skeletal muscle adheron (29). This is probably due to the cellular heterogeneity of the neural retina, for many cell types are present in the tissue (21).

Although the protein composition of the chick neural retina cells is heterogeneous, the size of the particles as determined by electron microscopy is quite homogeneous. The mean diameter size is 15.4 ± 2.0 nm, a standard error well within the range found using similar techniques applied to purified protein preparations (32). Although the morphology of the particle was largely spherical, an occasional structure was observed which had a spherical core and radial arms (Fig. 7 *C*). This sun-burst or pinwheel structure is similar to that observed for a sponge aggregation factor (10). The similarity between the adhesive activities of skeletal muscle adherons and sponge factors was previously noted (29).

Particles similar in size to the chick neural retinal adherons have been described in vivo and recently in cell culture. Particles between 10 and 40 nm in diameter were observed in corneal epithelial basement membrane following ruthenium red-osmium fixation, suggesting that they contain GAGs (9). More recently, an ultrastructural analysis of the extracellular matrix synthesized by endothelial cells showed that it consisted of 30-nm spherical "nodes" which are stacked in parallel arrays and separated by ~100 nm (13). The particles described above may be similar to neural retina adherons and involved in the adhesive interactions of cells with the extracellular matrix.

In addition to demonstrating that the 12S particles directly promote cell-substratum adhesion and increase the rate of cell aggregation, monovalent Fab′ fragments of antibodies prepared against the particles inhibit both cell adhesion to adheron-coated substrata and also the spontaneous aggregation of the cells. Furthermore, adherons neutralize the inhibition of aggregation by the antibody. Although the antiserum used here is not so active as that used by Thiery et al. (31), these immunological data show that antigenic determinants within the 12S particle are involved in the normal, spontaneous cell-cell interactions within cultured neural retina cells. One class of molecules that may be involved in cell-substratum adhesion are the GAGs, for heparin and heparan sulfate inhibit the initial rate of adhesion of cells to adheron-coated surfaces (Fig. 1). GAGs and proteoglycans have been implicated in the cell-substratum adhesion of other cell types (see, for example, references 4, 26, 28), including clonal nerve cell lines (5).

The adhesion between chick neural retina cells can be either calcium-dependent or calcium-independent, depending upon the assay system (7, 14, 30). Since the neural retina adheron-mediated adhesions do not require calcium, the particle-mediated system may be involved in the calcium-independent mechanism. Although chick neural retina adherons do not spontaneously aggregate in sucrose gradients, the fact that the 12S particle can be quantitatively removed from the culture medium by a 3-h centrifugation at 100,000 *g* indicates that the particles are aggregated in growth conditioned medium. The mechanism of this particle aggregation is unknown. Assuming that sucrose does not directly inhibit particle aggregation, these data are compatible with the possibilities that neural retina adherons cause cell aggregation by binding simultaneously to two cells, forming a single particle bridge, or that adherons on the surfaces of pairs of cells interact with each other in the presence of an unknown cofactor. In the case of myoblasts, adherons bind to the surface of cells in a calcium-independent manner, and the surface-bound particles interact with each other in the presence of calcium to cause cell aggregation (29). Since the adherons of both neural retina and muscle bind directly to plastic substrata, the interaction between the immobilized particle and the cell could lead to an increased rate of cell-substratum adhesion.

This work was supported by grants from the Muscular Dystrophy Association of America and the National Institutes of Health to D. Schubert.

Received for publication 8 October 1982, and in revised form 4 January 1983.

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