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Distinct calcium-independent and calcium-dependent adhesion systems of chicken embryo cells

(cell aggregation/retinal cells/hepatocytes/morphogenesis)

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ABSTRACT **Three criteria have been used to distinguish among different systems of embryonic cell adhesion: dependence on Ca^{2+}, involvement of particular cell-surface molecules, and binding specificity. The characterization of the adhesion with respect to cell-surface molecules was carried out by using specific antibodies against the neural and liver cell adhesion molecules (N-CAM and L-CAM) and antibodies raised against retinal cells prepared by limited trypsinization in the presence of Ca^{2+} (called "T/Ca cells"). Aggregation of cells prepared from retina or brain without Ca^{2+} did not require Ca^{2+} and was inhibited by anti-(N-CAM) antibodies but not by anti-(L-CAM) or anti-T/Ca cell antibodies. In contrast, cells obtained from the same tissues in the presence of Ca^{2+} did require Ca^{2+} to aggregate. This aggregation was inhibited by anti-T/Ca cell antibodies but not by anti-(N-CAM) or anti-(L-CAM) antibodies. Hepatocyte aggregation also required Ca^{2+} and was inhibited only by anti-(L-CAM) antibodies. These results define three antigenically distinct cell adhesion systems in the embryo and raise the possibility that additional systems will be found.**

The neural Ca^{2+}-independent system displayed a limited tissue specificity, mediating binding to neural but not liver cells. In contrast, the Ca^{2+}-dependent systems of both neural and liver cells caused binding to all cell types tested. The Ca^{2+}-dependent system was most active in retinal cells from 6–7 day embryos, whereas the Ca^{2+}-independent system was most active at later times during development. In addition, treatments that inhibited the Ca^{2+}-independent or Ca^{2+}-dependent systems had very different effects on the fasciculation of neurites from dorsal root ganglia. All of the results suggest that Ca^{2+}-independent and Ca^{2+}-dependent adhesion systems play different functional roles during embryogenesis.

Many developmental events require cell–cell interactions that presumably are mediated by binding events between specific cell-surface molecules. These interactions take place in a complex setting consisting of a variety of cell types in which different processes such as cell division, differentiation, motility, and death are occurring simultaneously (1, 2). Although there is general agreement that an analysis of the molecular basis of cell–cell interaction requires simplified *in vitro* assays, there is less agreement on the appropriateness of methods to be used. The *in vitro* aggregation of cells from different chicken embryo tissues has been studied in several laboratories; although several molecules and activities (3–8) have been implicated in adhesion, no definitive overall picture of the mechanism of cell-to-cell binding has emerged.

In previous studies in chicken embryos, we have examined the aggregation of neural cells prepared by trypsinization and allowed to recover in suspension culture (9, 10); we also have studied liver cells that were dispersed by a more limited protease treatment (11). So far, two molecules have been identified (3, 11), the function of which is necessary for the *in vitro* aggregation of these cells. These have been named neural cell adhesion molecule (N-CAM) and liver cell adhesion molecule (L-CAM) (11). The two CAMs are antigenically distinct.

The present study is concerned with a comparison of the aggregation of retina, brain, and liver cells prepared by different tissue-dissociation techniques (10–13) that have been shown to give calcium-dependent and calcium-independent adhesion (13–18). We have examined these cell types with respect to the involvement of particular cell adhesion molecules in the binding process, and the ability of the different cell types to bind to each other. The results suggest the presence of at least three distinct adhesion systems during development: a calcium-independent and a calcium-dependent system in neural cells and a different calcium-dependent system in liver cells. These findings are related to studies by other workers and are discussed in terms of their significance in organogenesis.

MATERIALS AND METHODS

Preparation of Cells. Liver cells were dissociated from 11-day chicken embryo tissue using collagenase, trypsin, and bovine serum, as described (11, 19). Retina and brain cells were prepared from tissue by three different procedures: (*i*) "T/SC," extensive trypsinization [with 0.5% trypsin (1:250; GIBCO) in Ca^{2+}-free medium for 20 min at 37°C] followed by culture in suspension for 20 hr (10); (*ii*) "T/Ca," limited trypsinization [with 0.04% twice-recrystallized trypsin (Worthington) for 25 min at 37°C] in Hepes-buffered, Ca^{2+}/Mg^{2+}-free medium (14) supplemented with 10 mM $CaCl_2$; and (*iii*) "T/E," limited trypsinization (with 0.002% twice-recrystallized trypsin for 15 min at 37°C) in Hepes-buffered, Ca^{2+}/Mg^{2+}-free medium containing 1 mM EDTA. The latter two protocols were slight modifications of methods described by Urushihara *et al.* (13). In all cases, the tissue was triturated briefly after 5 min of treatment with enzyme and was dispersed into single cells with a pasteur pipette at the end of the digestion. Cells were pelleted (700 × *g*, 4 min) through 3.5% bovine serum albumin in medium onto a cushion of 35% (vol/vol) isotonic albumin (Miles Laboratories) and then washed twice in Hepes-buffered, Ca^{2+}/Mg^{2+}-free medium. The viability of the single-cell preparations was 90% or better as determined by trypan blue exclusion.

Antibodies. In all experiments, monovalent Fab' fragments prepared from the IgG fraction of rabbit serum (10) were used. Antibodies against purified N-CAM and L-CAM were prepared as described (3, 11). These two antibodies have been shown to have different specificities (11). Polyspecific antibodies (anti-T/

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Abbreviations: N-CAM, neural cell adhesion molecule; L-CAM, liver cell adhesion molecule; T/SC, extensive trypsinization in Ca^{2+}-free medium followed by culture in suspension; T/E, limited trypsinization in EDTA; T/Ca, limited trypsinization with Ca^{2+}

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Ca cell) that inhibit Ca^{2+}-dependent adhesion of retina cells were produced by intramuscular, intraperitoneal, and subcutaneous injections of rabbits with 5×10^7 retinal T/Ca cells. The first injection was in complete Freund's adjuvant, followed by injections in incomplete Freund's adjuvant at 2-wk intervals. Bleedings followed injections by 7 days, and antibodies of adequate titer were obtained after four injections. Control Fab' was prepared from serum of rabbits injected only with adjuvant.

Cell–Cell Adhesion Assays. Aggregation of cells in suspension was measured by determining the rate of decrease of particle number as described (10). Hepes-buffered, Ca^{2+}/Mg^{2+}-free medium supplemented with either 10 mM $CaCl_2$ or 1 mM EDTA was added to 5×10^6 neural cells or 2×10^6 liver cells to a final volume of 2 ml. In some experiments, the cells were preincubated (15 min at 4°C) in 0.2 ml of Fab' (5 mg/ml). Cell suspensions were shaken (90 rpm; 37°C for neural cells, room temperature for liver cells) in scintillation vials. Aliquots removed at 0, 20, and 40 min were diluted into phosphate-buffered saline containing 1% (vol/vol) glutaraldehyde, and then cells were counted using a Cytograph (Ortho Diagnostic Systems, Westwood, MA). Rates of aggregation were expressed as the percent reduction in particle number after a 20- or 40-min interval.

The monolayer cell–cell binding assay (20, 21) was used to quantitate binding between dissimilar cell types. To maximize cell yield for these experiments, T/E neural cells were prepared from 10-day retinas and T/Ca neural cells from 7-day brains. Cells were pelleted in graduated tubes and resuspended in 10 vol of Hepes-buffered medium containing $CaCl_2$. These cells (2 ml) were labeled internally by adding 0.5 ml of fluorescein diacetate (10 μg/ml) and incubating for 10 min at room temperature. The cells were washed twice and resuspended to 1×10^8 cells per ml. Cell monolayers were prepared in 35-mm petri dishes (Falcon), which had been pretreated for 30 min with a solution of waxbean agglutinin (22) in phosphate-buffered saline (100 μg/ml) and then rinsed. For each dish, 0.6 ml of the 10% (vol/vol) cell suspension was added to 3.4 ml of medium, transferred into the treated petri dish, and centrifuged 4 min at $300 \times g$. When indicated, monolayers and suspension cells were preincubated for 10 min in 0.4 ml of medium containing Fab' fragments of antibodies (5 mg/ml). Suspension cells were added to the monolayer in 5 ml (final volume) of medium and incubated at 60 rpm in a New Brunswick Environmental Incubator for 30 min at room temperature. Unbound cells were removed by washing, and the number of fluorescent cells bound to monolayer cells was determined by counting 20 fields at ×400 magnification using a fluorescence microscope.

Dorsal Root Ganglia Cultures. Spinal ganglia from 9-day chicken embryos were placed in 35-mm plastic culture dishes containing 2 ml of medium and 50 ng of 2.5S nerve growth factor per ml as described (23). The media used were minimal essential medium with and without 10 mM Ca^{2+}. Antibodies (control or anti-T/Ca cell) were dialyzed against medium before use and were added after 17 hr of culture to a final concentration of 1.0 mg/ml. Cultures were observed by phase-contrast microscopy after 24 hr.

RESULTS

Ca^{2+}-Dependent and Ca^{2+}-Independent Aggregation of Retinal Cells Involve Antigenically Distinct Components. The aggregation properties of retinal cells prepared by three different enzymatic techniques are compared in Table 1. Cells obtained from 7- to 10-day-old embryos by extensive trypsinization did not aggregate until they had been allowed to recover for several hours in suspension culture (10). The aggregation of these cells (T/SC cells) did not require Ca^{2+} and was strongly inhibited by anti-(N-CAM) antibodies as reported (3). Cells dispersed by incubation with a low level of trypsin in the presence of EDTA (T/E cells) (13) were able to aggregate extensively within 40 min. As with suspension-cultured T/SC cells, the aggregation of these cells did not require Ca^{2+} and was strongly inhibited by anti-(N-CAM) antibodies. Cells prepared by trypsinization in the presence of 10 mM Ca^{2+} (T/Ca cells) (12, 13) also aggregated within 40 min. In contrast to the other two cell types, the aggregation of these cells required Ca^{2+} and was not significantly inhibited by preincubation with anti-(N-CAM) antibodies.

Table 1. Aggregation of retinal cells prepared by different techniques

Preparation*	Aggregation properties: Ca^{2+},† 10 mM	Aggregation rate‡	% inhibition by Fab': Anti-(N-CAM)	% inhibition by Fab': Anti-T/Ca cell
T/SC: 0.5% trypsin and culture in suspension	−	40	85	16
	+	39		
T/E: 0.002% trypsin with 1 mM EDTA	−	60	94	18
	+	62		
T/Ca: 0.04% trypsin with 10 mM Ca^{2+}	−	6		
	+	53	9	78

*Retinas from 7-day embryos were used to prepare T/Ca cells. Retinas from 10-day embryos were used to prepare T/SC and T/E cells.

† Hepes-buffered, Ca^{2+}/Mg^{2+}-free medium with 10 mM $CaCl_2$(+) or with 1 mM EDTA (−) was used.

‡ Expressed as the percent decrease in particle number after a 20 min incubation at 37°C.

Because antibodies against N-CAM blocked only the calcium-independent mode of aggregation, the antigenic relationships between this system and the calcium-dependent system were explored further. Antibodies raised against cells prepared by treatment with trypsin/10 mM Ca^{2+} (anti-T/Ca cells) were tested for their effect on aggregation (Table 1). As opposed to the results obtained with anti-(N-CAM) serum, the anti-T/CA cell serum strongly inhibited the Ca^{2+}-dependent aggregation but had a small effect on the Ca^{2+}-independent aggregation of retinal cells. In contrast to anti-(N-CAM) and anti-(L-CAM), which have defined specificities (11), anti-T/Ca cell antibodies are a polyspecific reagent whose adhesion-blocking activity has not been characterized. It has not been proven, therefore, that T/Ca cell aggregation occurs via specific cell-surface molecules. The antigenic distinction between the T/Ca and T/E cell adhesion systems was confirmed, however, by the fact that the Ca^{2+}-dependent adhesion-blocking activity of anti-T/Ca cell was removed by absorption with T/Ca cells but not reduced by absorption with T/E cells (data not shown).

Antigenic Comparison of Aggregation Systems from Retina, Brain, and Liver. Antigenic relationships between the Ca^{2+}-dependent and Ca^{2+}-independent aggregation systems of retina, brain, and liver were probed with anti-(N-CAM), anti-(L-CAM), and the polyspecific anti-T/Ca cell (Table 2). Liver cells required Ca^{2+} to aggregate as reported (24), and brain cells displayed the same Ca^{2+}-dependent properties as retinal cells. For each cell type, only one of the three antibody preparations substantially reduced aggregation. Anti-(N-CAM) antibodies strongly inhibited the Ca^{2+}-independent aggregation of cells from both retina and brain but had no effect on Ca^{2+}-dependent aggregation of neural or liver cells. Antibodies against L-CAM

Table 2. Aggregation of cells from different tissues

Cell preparation*	Tissue	Aggregation rate†	% inhibition by Fab' Anti-(N-CAM)	Anti-(L-CAM)	Anti-T/Ca cell
T/E	Retina	66	94	8	18
T/E	Brain	58	81	7	5
T/Ca	Retina	54	9	0	68
T/Ca	Brain	53	6	0	57
C/T/B‡	Liver	35	9	61	26

* See Table 1.

† Expressed as the percent decrease in particle number after a 20-min incubation at 37°C for retina and brain tissues and at 25°C for liver tissue. Hepes-buffered, Ca^{2+}/Mg^{2+}-free medium with 1 mM EDTA (for T/E cells) or with 10 mM $CaCl_2$ (for T/Ca and C/T/B cells) was used.

‡ Treatment with a mixture of collagenase, trypsin, and bovine serum (11, 19).

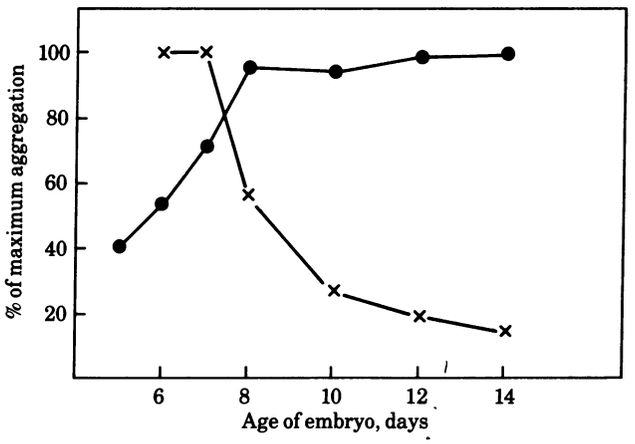

FIG. 1. Ca^{2+}-independent (●) and Ca^{2+}-dependent (×) aggregation of retinal T/E and T/Ca cells prepared from embryos of different ages. Percentage aggregation values after 30 min, similar to those shown in Table 1, were converted to percent of maximum values.

inhibited only the aggregation of liver cells, without affecting either Ca^{2+}-independent or -dependent systems of retina and brain. The antibodies raised against retina T/Ca cells inhibited Ca^{2+}-dependent aggregation of both retina and brain cells; they had significantly less effect on the Ca^{2+}-independent binding of retina and brain and the Ca^{2+}-dependent binding of liver cells.

Comparison of the Binding Specificities of the Neural and Liver Aggregation Systems. The adhesive specificity of each system was defined by studying the binding between the different cell types (heterotypic binding) in the monolayer cell–cell binding assay (Table 3). With all cell combinations, significant levels of binding were obtained, although in general heterotypic binding was half to two-thirds of the levels obtained with homotypic binding. In each case, the adhesion systems responsible for the binding were identified by determination of the Ca^{2+}-dependence and pattern of inhibition by antibodies.

The binding between liver cells and neural T/E cells was Ca^{2+}-dependent (Table 3). The binding was inhibited by anti-(L-CAM) but not anti-(N-CAM) antibodies, indicating that it is mediated solely by the L-CAM system. Therefore, T/E cells appear to have components capable of interacting with the liver adhesion system, whereas liver cells apparently lack receptors that can interact with the neural Ca^{2+}-independent system.

The binding of liver cells to neural T/Ca cells was Ca^{2+}-dependent, partially inhibited by either anti-(L-CAM) or anti-T/Ca cell antibody alone and strongly inhibited when both antibodies were present together. This result suggests that both the L-CAM and neural Ca^{2+}-dependent systems participate in the binding. Consequently, each of these cell types appears to have components capable of interacting with the Ca^{2+}-dependent adhesion system of the other cell type.

In the experiments combining liver with either T/E or T/Ca cells, reversal of the suspension and monolayer cell types gave identical results. However, this was not the case in binding between T/Ca and T/E cells (Table 3). When the monolayer was composed of T/Ca cells, the binding of T/E cells was Ca^{2+}-dependent and inhibited only by anti-T/Ca cell antibodies. When T/E cells were in the monolayer, the binding of T/Ca cells was Ca^{2+}-independent and was inhibited only by anti-(N-CAM) antibodies. This unexpected finding suggests that the attachment of cells to substratum can in some cases affect their surface membrane properties, but these effects were not investigated further.

Neural Ca^{2+}-Dependent and Ca^{2+}-Independent Systems Are Active at Different Times During Development. To indicate the time during development at which Ca^{2+}-dependent and Ca^{2+}-independent systems might operate, cells of each type were prepared from embryos of different ages, and their rates of aggregation were determined. As shown in Fig. 1, the amount of aggregation varied as a function of age for both cell types, but the profiles were different. Ca^{2+}-dependent aggre-

Table 3. Binding between cells having different adhesion systems

Heterotypic binding between: Cell in monolayer	Cell in suspension	Ca^{2+}, 10 mM	Number of cells bound to monolayer*	% inhibition by Fab'† Anti-(N-CAM)	Anti-(L-CAM)	Anti-T/Ca cell	Anti-(L-CAM) plus anti-T/Ca cell
Liver C/T/B‡	Neural T/E	+	202	0	63		
		−	58				
Liver C/T/B‡	Neural T/Ca	+	402		39	32	73
		−	130				
Neural T/Ca	Neural T/E	+	216	0		75	
		−	21				
Neural T/E	Neural T/Ca	+	266	80		15	
		−	259				

* Twenty microscope fields representing a total area of 1 mm^2 were scored.

† In cases where Ca^{2+}-dependent binding was obtained, the percentages shown were calculated after subtraction of the binding obtained in the absence of Ca^{2+}.

‡ Cells treated with a mixture of collagenase, trypsin, and bovine serum (11, 19).

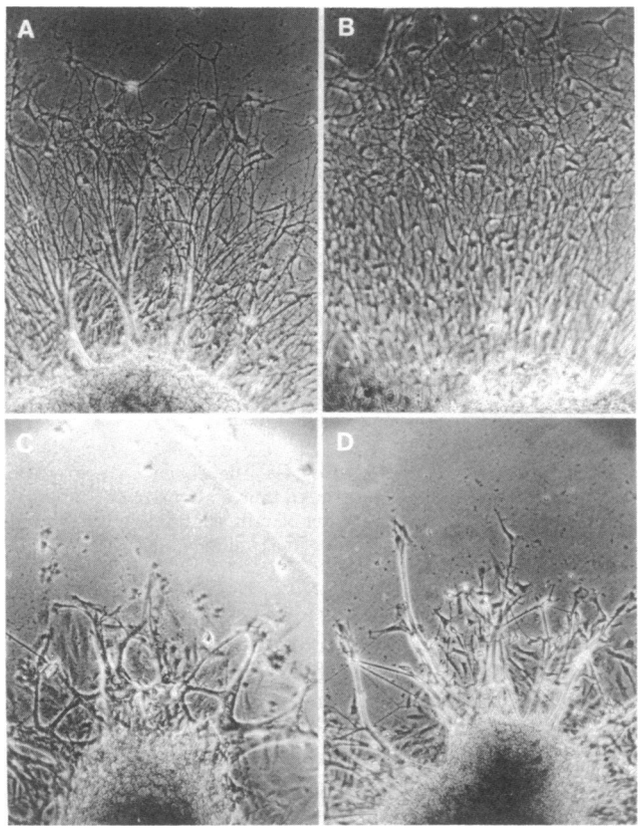

FIG. 2. Effect of treatments that inhibit neural Ca^{2+}-dependent and Ca^{2+}-independent binding on the formation of neurite bundles by dorsal root ganglia in culture. (*A*) Control ganglion cultured in the presence of Ca^{2+} and Fab' from unimmunized rabbits. (*B*) Ganglion cultured in the presence of anti-(N-CAM) Fab' fragment (1 mg/ml). (*C*) Ganglion cultured in the absence of Ca^{2+}. (*D*) Ganglion cultured in the presence of anti-T/Ca Fab' fragment (1 mg/ml). (×140.)

gation was most effectively detected with T/Ca retinal cells from 6–7 day embryos; T/Ca cells prepared from older embryos aggregated poorly. In contrast, aggregation due to the Ca^{2+}-independent system was lower in T/E cells from 6- to 7-day embryos and increased to a higher level in cells from 8- to 14-day embryos. Whereas T/E cells from 14-day embryos retained the ability to aggregate, T/SC cells prepared from these embryos aggregated poorly and had little N-CAM on their surfaces (25). Therefore, although N-CAM on retinal cells is preserved during limited trypsinization, it is removed by a more extensive trypsinization and is not regenerated by cells from older embryos.

Neurite Fasciculation Is Affected Differently by Inhibition of the Ca^{2+}-Independent and Ca^{2+}-Dependent Systems. Antibodies against N-CAM reduce the thickness of neurite fascicles formed in cultures of dorsal root ganglia, suggesting that the Ca^{2+}-independent system is involved in side-to-side adhesion of neurites (23). To test for a possible role of the Ca^{2+}-dependent system in this process, the fasciculation of dorsal root ganglia neurites after 24 hr of culture was compared in the presence and absence of Ca^{2+} or anti-T/Ca cell antibodies. In contrast to the results obtained with antibodies against N-CAM (Fig. 2*B*; ref. 23), conditions which reduced Ca^{2+}-dependent adhesion, such as removal of Ca^{2+} or addition of anti-T/Ca cell antibodies, increased the thickness of fascicles formed (Fig. 2 *C* and *D*). However, because anti-T/Ca cell antibody is not monospecific, it remains to be determined whether its effect on fasciculation is directly related to its inhibition of Ca^{2+}-dependent adhesion.

DISCUSSION

The present studies provide evidence for the existence of three different systems of adhesion among embryonic chicken cells. These systems differ not only in the antigenic properties of the molecules involved but also in their dependence on Ca^{2+}, specificity of binding, and expression during development. The following discussion summarizes the basis for these conclusions and considers some implications for their role in cell adhesion during development.

In previous work (3, 9, 10), we studied the aggregation of retina and brain cells prepared by extensive trypsinization followed by culture in suspension. These cells appeared to aggregate via a single mechanism involving the neural cell adhesion molecule N-CAM. Recent studies using different cell preparation techniques (13, 14, 16, 18) have suggested that retinal cells can aggregate by two different modes that are distinguished by their requirement for Ca^{2+}. The present study used antisera to provide direct evidence supporting the suggestion (14, 16) that these two modes result from distinct mechanisms. Our experiments showed that N-CAM is involved in Ca^{2+}-independent aggregation and that Ca^{2+}-dependent aggregation involves different but as yet unidentified molecules. In addition, we have shown that the liver cell adhesion molecule L-CAM, which is involved in Ca^{2+}-dependent adhesion and is structurally distinct from N-CAM (11), is also antigenically unrelated to the neural Ca^{2+}-dependent system.

Another issue addressed by the present work was the degree of binding specificity displayed by these three types of adhesion. Although the Ca^{2+}-dependent systems of neural and liver cells involved different molecules, they displayed the same broad specificity in binding to different cell types. In contrast, the neural Ca^{2+}-independent system had a more restricted specificity in that it mediated binding to neural but not to liver cells.

The difference in the binding specificity of the two neural systems suggests that they have different functions. This possibility is supported by the observation that in retina the Ca^{2+}-dependent system was most active with cells derived from 6- to 7-day embryos, whereas the Ca^{2+}-independent system was most active with embryos 8–14 days of age. Furthermore, the period of changeover from one system to the other occurs at the time when cell division ceases and the formation of cell body and plexiform layers begins. Therefore, the apparent complementarity between the two systems may reflect a functional interrelatedness or cooperation during this transition.

The Ca^{2+}-independent N-CAM system has been associated with several aspects of neural development, including the formation of neurite bundles (23), the sorting out of neurites and somata in cell aggregates (25), and the organization of cell layers in retinal tissue (26). Although no function has as yet been assigned to Ca^{2+}-dependent aggregation, treatments that inhibit this system caused an increase in the thickness of neurite fascicles in culture. This observation is opposite to the effect obtained with antibodies against N-CAM and could have been produced by a decrease in the strength of adhesion between growth cones and substratum (27). Investigation of a possible role for Ca^{2+}-dependent adhesion in cell–substrate adhesion will require both the identification and isolation of molecules involved in this system and the preparation of specific adhesion-blocking antibodies.

In view of the present findings, it is likely that an extension of these studies to other tissues will reveal other antigenically distinct but perhaps functionally related adhesion systems. Moreover, the results obtained with the two neural systems raise the possibility that formation of cell patterns in a particular

tissue could reflect a sequence or cascade of several different adhesion mechanisms involving different molecules.

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