Journal of Supramolecular Structure
and Cellular Biochemistry 16:259-268 (1981)
Cellular Recognition 253-262

Molecules Involved in Cell–Cell Adhesion During Development

Gerald M. Edelman and Urs Rutishauser
The Rockefeller University, New York, New York 10021

Over the past decade there have been many attempts to identify the molecules that serve as ligands in cell–cell adhesion. In general, these studies have focused on a search for substances endogenous to a tissue that can either enhance or inhibit aggregation directly and that either demonstrate tissue specificity or are present in concentration gradients. Although in many instances the same cell types have been studied, there has been scant agreement on the molecular basis of cell–cell binding.

Informed and perhaps prejudiced by the immunological interests of our laboratory, we have developed a somewhat different approach to the identification of molecules involved in adhesion. These studies utilize an immunological assay for cell-adhesion molecules based on the inhibition of adhesion by antibodies prepared against whole cells and the identification of antigens recognized by these antibodies. This procedure has several advantages: it does not require assumptions about the number, composition, or mode of action of cell-adhesion molecules, and, provided they retain their antigenicity, it does not require that they retain their biological activity after their removal from the cells of origin. In the following sections, we describe this assay [1], its use in purifying from chick embryo retinal tissue a molecule associated with neural cell adhesion [2], the production of antiserum that inhibits nerve cell aggregation [2], and the use of this antiserum to probe some of the physiological consequences of cell–cell adhesion [3–5]. This experimental approach has also been used to explore the molecular basis of other cell-adhesion systems [6,7], and they are also considered briefly.

THE ASSAY AND PURIFICATION OF A MOLECULE FROM NEURAL RETINA

The development of a sensitive and specific assay for molecules involved in cell adhesion required the application of particle-counting methods for quantitating cell adhesiveness [8], the preparation of antibodies that specifically inhibit cell adhesion, and the preparation and partial fractionation of antigens

Received June 1, 1981; accepted June 9, 1981.

0275-3723/81/1603-0259$03.00

from retinal cells that can neutralize these antibodies. To obtain antibodies that can inhibit cell adhesion, rabbits were immunized with cells from retinas of 10-day-old chick embryos (anti-R10), and Fab′ fragments were prepared from the antibodies. Being monovalent, the Fab′ fragments did not cause agglutination, but instead inhibited the aggregation of the retinal cells (Fig. 1).

The immunoassay for molecules involved in cell adhesion consists of

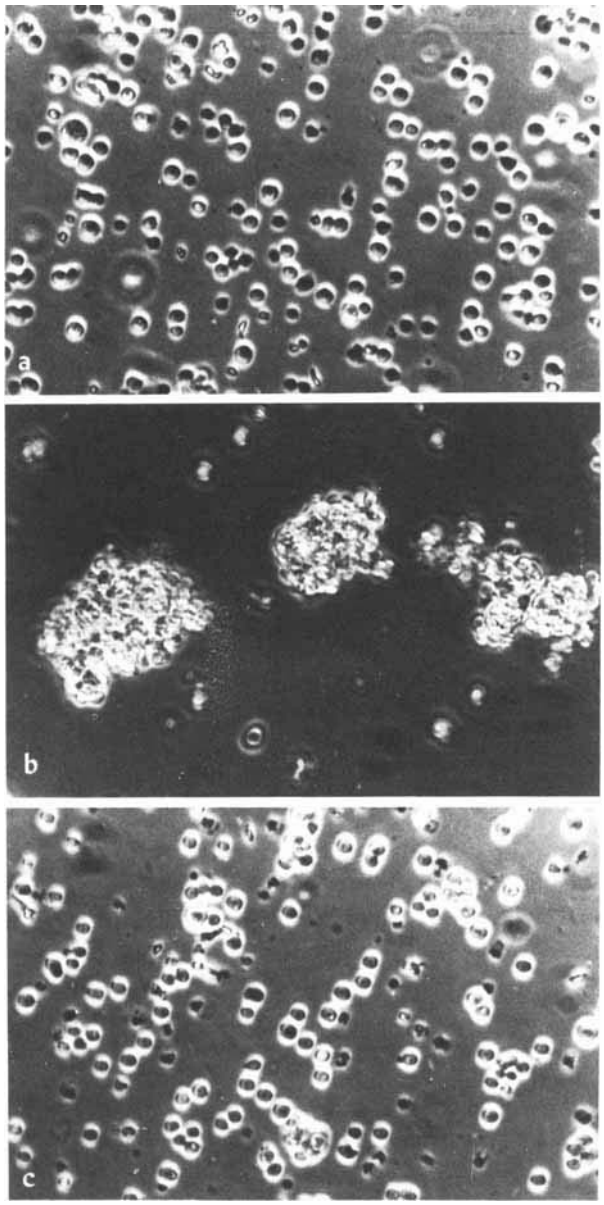

Fig. 1. Aggregation of retinal cells from 10-day-old chick embryos. (a) Cells prior to aggregation. (b) Aggregates produced after incubation for 30 min at 37°C. (c) Aggregation for 30 min at 37°C in the presence of anti-R10 Fab′ (Brackenbury et al [1]).

measuring the ability of particular retinal cell antigens to neutralize the adhesion-inhibiting activity of anti-R10 Fab', thus permitting aggregation to occur [1]. Supernatants from 24-hour cultures of intact 10-day retinal tissue (TCS) in serum-free medium were found to be a convenient source of such neutralizing antigens. Although the retinal-cell molecules present in the TCS did not themselves block or enhance aggregation, they did reverse the inhibition by the anti-R10 Fab' (Table I). The amount of neutralization, expressed as percent decrease in inhibition, was linearly dependent on the logarithm of the supernatant volume added over a reasonable range of dilutions. It was therefore possible to estimate the relative amount of neutralizing antigen in a sample by its effect on the aggregation of cells in the presence of a constant amount of Fab'. For purposes of quantitation, one unit of neutralizing activity was defined as the amount of antigen needed to cause a 25% decrease in the inhibition of adhesion produced by 1 mg of Fab' fragment from the reference antiserum (anti-R10).

To purify the molecules responsible for the neutralization of anti-R10 Fab' by TCS, their neutralizing activity was monitored throughout a fractionation of the TCS mixture by gel filtration and polyacrylamide gel electrophoresis [2]. These fractionations resulted in a 500-fold increase in specific neutralizing activity (Table II). This partially purified antigen consisted of a polypeptide with

TABLE I. Effect of Tissue-Culture Supernatant and Anti-R10 Fab' on Aggregation of Cells From 10-Day Retinas*

Assay	Anti-R10 Fab' (mg/assay)	Tissue culture supernatant (μl)	Aggregation (Δ%)[a]
Aggregation	0	0	41 ± 2
Effect of TCS	0	5-100	39 ± 2
Inhibition by Fab'[b]	1	0	18 ± 1
Neutralization of Fab' by TCS	1	50	33 ± 2

*The standard deviation in triplicate assays is shown.
[a]Percent decrease in particle number after 20 min.
[b]Neutralizing activity is most reliably measured when the amount of Fab' added causes about a 50% inhibition of cell aggregation.
Data from Brackenbury et al [1].

TABLE II. Fractionation of Activity From Tissue-Culture Supernatants

Fraction	Activity (units)	Protein (μg)	Specific activity	Activity yield (%)
Total TCS (400 retinas)	1,622	30,000	0.054	100
Activity recovered following gel filtration	1,151	2,600	0.44	71
Polyacrylamide gel electrophoresis	811	29	28.0	50

[a]Based on optical absorbance, with 1 mg/ml protein equal to 1.0 absorbance at 280 nm.
Data from Thiery et al [2].

an apparent molecular weight in SDS of 150,000; some smaller fragments of this molecule were also present. Immunoprecipitation studies revealed that the same antigen is present on the cell surface membrane. We have named this component neural cell adhesion molecule, or N-CAM.

PREPARATION AND CHARACTERIZATION OF ANTI-(N-CAM) ANTIBODIES

To obtain antibodies against N-CAM, rabbits were immunized with the active fraction from polyacrylamide gel electrophoresis. As shown in Table III, monovalent Fab′ fragments prepared from anti-(N-CAM) antibodies inhibit binding between retinal cells as well as between brain cells. This observation is consistent with the fact that N-CAM is present in many neural tissues [3], particularly in regions that are rich in neurites such as the plexiform layers of developing retina. To examine the possibility that N-CAM is involved in certain cell-adhesion phenomena previously described by other workers, the Fab′ of anti-(N-CAM) was tested for its ability to inhibit both homologous and heterologous adhesion between cells from dorsal and ventral regions of the retina [9,10], binding of membrane vesicles to retinal cells [11], and aggregation of liver cells [12,13] (Table III). In all the experiments with neural cells, initial adhesion was strongly inhibited by anti-(N-CAM) Fab′. In contrast, aggregation of liver cells was not affected by the antibody fragment.

EFFECT OF ANTI-(N-CAM) FAB′ ON HISTOTYPIC DEVELOPMENT OF RETINAL CELL AGGREGATES AND TISSUE

The presence of N-CAM in plexiform layers formed in the retina suggested that it might function during their development. To explore this hypothesis, we first carried out experiments in vitro on histotypic development of retinal cell aggregates [3] and tissue [5], particularly in relation to the sorting out of cells and neurites.

TABLE III. Effect of Anti-(N-CAM) Fab′ on Cell Adhesion

Binding between[a]			Binding[b]	
Cell A	Cell B	Assay used	Fab′ from unimmunized rabbits (%)	Anti-(N-CAM) Fab′ (%)
Retina	Retina	Monolayer	33	4
Brain	Brain	Monolayer	30	2
Retina	Brain	Monolayer	32	4
Dorsal retina	Dorsal retina	Monolayer	33	2
Ventral retina	Ventral retina	Monolayer	17	3
Dorsal retina	Ventral retina	Monolayer	51	8
Retina	Retinal membrane vesicles	Centrifugation	8.2	1.4
Liver	Liver	Particle counter	31	33

[a]Brain, retinal and liver cells from 6-, 8-, and 10-day-old embryos, respectively.
[b]Expressed as the percentage of cells in suspension bound to the monolayer, percentage of vesicles bound to cells, or the percent decrease in particle number after 20-min incubation.
Data from Rutishauser et al [3].

When aggregates of 8-day-old cells are maintained in culture, large regions containing neurites and synapses are formed over an interval of 7–8 days in a manner that resembles the differentiation of an intact retina [14,15]. After transfer of the aggregates that had been formed in culture over a 1-day period to medium containing 0.5–1 mg/ml anti-(N-CAM) Fab′ fragments, the neurite regions that subsequently appeared were much smaller, although the total amount of neuropil did not appear to be drastically decreased (Fig. 2). Aggregates cultured in medium containing 0.5–5 mg/ml Fab′ from unimmunized rabbits or from rabbits immunized with chick fibroblasts were indistinguishable from those grown without Fab′.

A more direct indication of the role of N-CAM in retinal development was revealed by modified organ cultures [5]. Cultures of 6-day retinal tissue on Millipore filters were found to display many features of normal development (Fig. 3). When these cultures contained anti-(N-CAM), however, several alterations

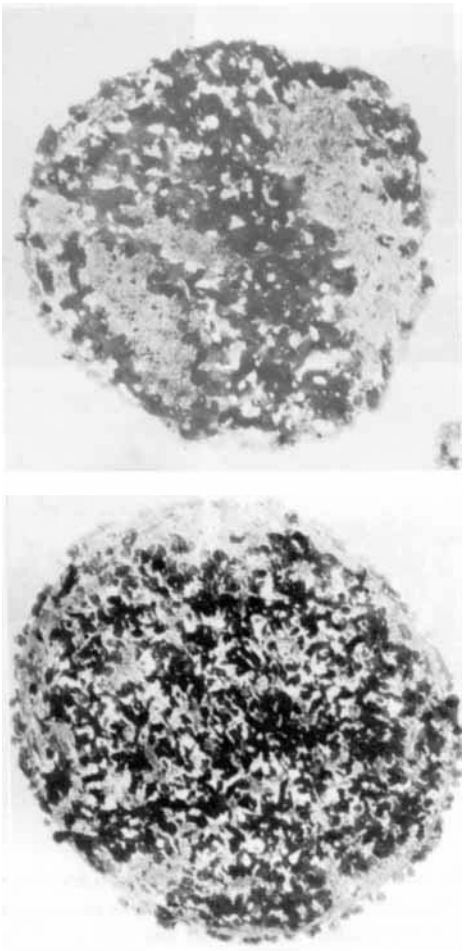

Fig. 2. Histology of retinal cell aggregates after culture for 7–8 days. Top: Aggregates that have formed large neurite regions after culture in medium containing 1 mg/ml Fab′ from unimmunized rabbits. Bottom: Aggregates that have been cultured in 1 mg/ml anti-(N-CAM) Fab′ (from Rutishauser [3]).

were observed. The ganglion cells, normally arranged in an even layer along the vitreous edge of the retina, were less organized and were found scattered throughout the inner plexiform layer. In control retinae, cells within the nuclear layer could be divided into two layers based on criteria related to cell shape, the outermost sublayer consisting of bipolar and horizontal cells and the sublayer toward the vitreous side of the nuclear layer corresponding to the ama-

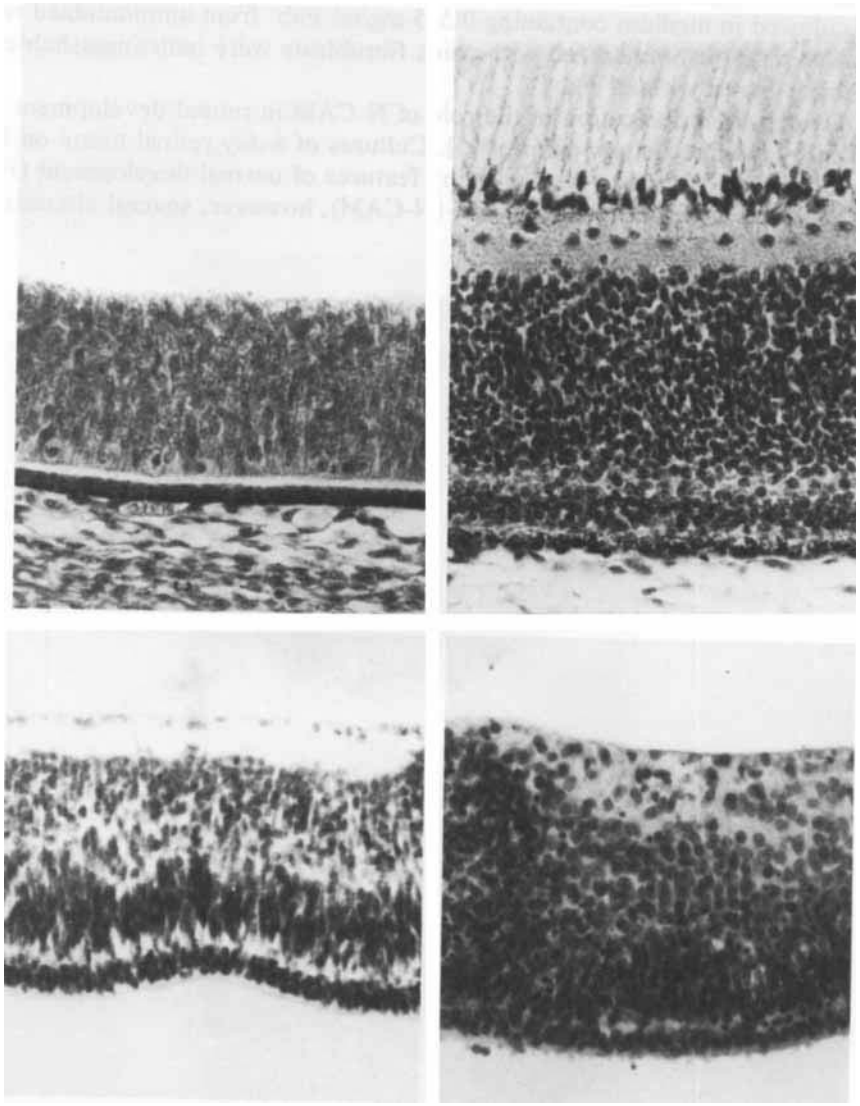

Fig. 3. Top, left and right represent in vitro chick retinae at 6 and 9 days of embryonic age, respectively. The photoreceptor cells are at the bottom in these photographs; at 9 days the outer and inner plexiform layers are clearly visible. Bottom, left and right: chick retinae that have been in organ culture for 3 days following their dissection from the embryo on day 6. The retina at the bottom left was cultured in the presence of F(ab′) antibody fragments from unimmunized rabbits, and the retina at the bottom right in the presence of antibodies to the cell-adhesion molecule N-CAM (from Buskirk et al [5]).

crine cells. In the retinae exposed to anti-(N-CAM), these layers were not clearly distinguishable, suggesting that the distribution of specific cell types had been altered (Fig. 3).

EFFECT OF ANTI-(N-CAM) ON NEURITE FASCICULATION

Electron microscopic studies of both retinal cell aggregates and the cultured tissue also suggested that the antibodies caused a reduction in the number of membrane–membrane contacts, particularly in regions consisting entirely of neurites. This effect on neurite interaction was further explored in spinal ganglia [4]. When dorsal root ganglia are cultured in the presence of nerve growth factor, a halo of processes and fascicles (bundles of processes) appears around each ganglion over a 24-hour period. In the presence of anti-(N-CAM), the morphology but not the extent of neurite outgrowth was altered (Fig. 4). The most obvious change was a decrease in the number of fascicles having a large diameter, accompanied by an increase in the number of processes consisting of one or a few neurites. This observation, along with the cinematographic studies revealing that anti-(N-CAM) enhances the splitting apart of bundles upon contact with the substratum, strongly suggested that N-CAM function is essential for neurite–neurite adhesion.

STUDIES OF OTHER CELL ADHESION SYSTEMS

The procedures developed to study neural cell adhesion have also been used to detect, purify, and characterize a cell surface component involved in the reaggregation of embryonic liver cells [6]. Antibodies prepared against this component, which we have called liver cell adhesion molecule or L-CAM, were used to probe the mechanism of liver cell adhesion, compare it to adhesion among embryonic neural cells, and investigate the role of L-CAM in the formation of histotypic liver cell colonies. Starting from liver plasma membranes, an 80-fold purification of L-CAM was achieved via a four-step fractionation. This purification allowed the production of antibodies of restricted specificity that inhibit liver cell aggregation. Using Fab′ fragments from these antibodies, we observed tha the appearance of histotypic liver cell patterns in culture was altered. Embryonic hepatocyes (cultured on plastic dishes) formed colonies with extracellular channels that had structural similarities to bile canaliculi [16]. When Fab′ from antibodies inhibiting liver cell aggregation was included in the medium, however, the cultures assumed a different morphology. Most conspicuous was the inability of the cells to associate into compact, three-dimensional colonies. Instead, they flattened out onto the substrate to form a monolayer; in the absence of colonies, both the size and the shape of the cells were altered, and specialized channels did not appear. Nonetheless, the cells remained viable in the cultures and their cytoplasmic and nuclear characteristics did not seem to be grossly changed (Fig. 5).

In addition to the discovery of CAMs from various tissues, the approach we have described is useful in dissecting the ion dependence of adhesion as first suggested by Takeichi [17].

Using two methods of tissue dissociation ("TE", 0.5% trypsin with 1 mM

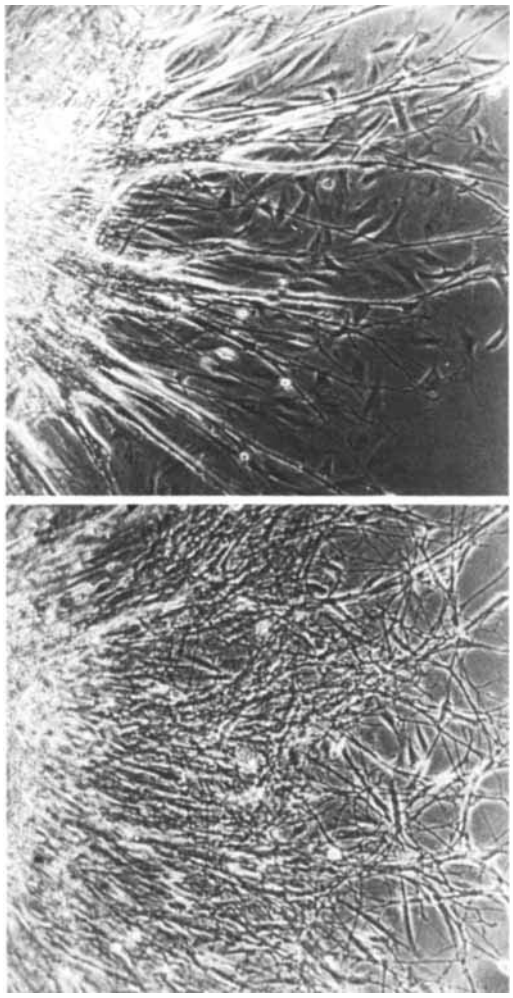

Fig. 4. Representative outgrowth of neurites from thoracic ganglia cultured for 24 h in medium containing 1 mg/ml Fab' from unimmunized rabbits (top) and 1 mg/ml anti-(N-CAM) Fab' (bottom). Ganglia cultured in medium containing 1–5 mg/ml anti-fibroblast Fab' were identical shown at the top. The presence of anti-(N-CAM) Fab' has resulted in a tangled outgrowth of fine processes rather than the thick and relatively straight fascicles formed in cultures without this antibody (from Rutishauser [4]).

EDTA; and "TCa," 0.04% trypsin with 10 mM Ca^{2+}) it is possible to prepare neural cells whose aggregation is independent or dependent on the presence of Ca^{2+} [17–20]. By testing the ability of anti-(N-CAM) and anti-(L-CAM) to inhibit aggregation, we have shown that the Ca^{2+}-independent mechanism involves N-CAM, and that the Ca^{2+}- and L-CAM-dependent aggregation of liver cell is antigenically distinct from the Ca^{2+}-dependent aggregation of neural cells (Table IV). In addition, antibodies prepared against the TCa-treated cells were found to inhibit CA^{2+}-dependent adhesion among neural cells but not adhesion

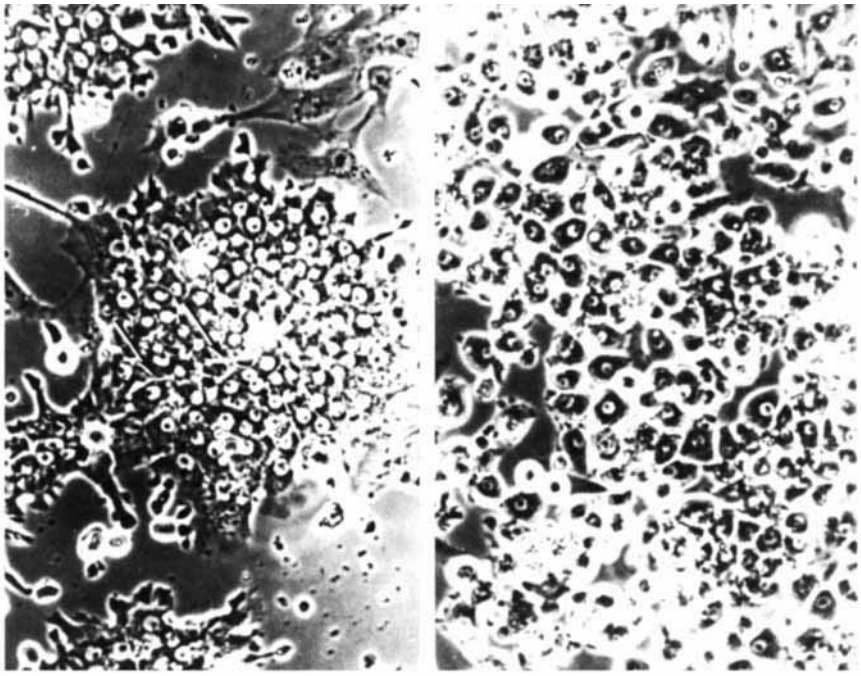

Fig. 5. Phase-contrast photomicrographs, at the same magnification, of 24-h hepaocyte cultures in medium supplemented with 10% (vl/vl) fetal calf serum: (Left) control with 0.5 mg of normal rabbit Fab′ per ml; (Right) with 0.5 mg anti-(L-CAM) Fab′ per ml. Compare the compact architecture of the control colony, with possible intercellular channels (arrows), with the flattened and unorganized monolayer obtained in the presence of the anti-(L-CAM) (from Bertolotti et al [6]).

TABLE IV. Aggregation of Cells From Different Tissues

		% inhibition by Fab′		
Cell preparation[a]	Aggregation rate[b]	Anti-(N-CAM)	Anti-(L-CAM)	Anti-TCa cell[c]
Neural TE	66	94	8	18
Neural TCa	54	9	0	68
Liver	35	9	61	26

[a]See text.

[b]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 TE cells) or with 10 mM $CaCl_2$ (for TCa and liver cells) was used.

[c]Antibodies raised in rabbits against chick retina cells prepared using trypsin in the presence of Ca^{2+} (see text).

Data from [7].

involving L-CAM or N-CAM. The Fab'-neutralization assay is now being used to identify a CAM involved in cell aggregation of TCa cells.

An extension of these approaches to other tissues will, in all probability, 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. One of the key goals of the analysis of cell adhesion is therefore to isolate the various CAMs in sufficient purity and amount to characterize precisely their binding mechanisms and molecular structure.

ACKNOWLEDGMENTS

This work was supported by U S. Public Health Service grants AI-11378, AM-04256, and HD-09635.

REFERENCES

1. Brackenbury R, Thiery J-P, Rutishauser U, Edelman GM: J Biol Chem 252:6835–6840, 1977.
2. Thiery JP, Brackenbury R, Rutishauser U, Edelman GM: J Biol Chem 252:6841–6845, 1977.
3. Rutishauser U, Thiery J-P, Brackenbury R, Edelman GM: J Cell Biol 79:371–381, 1978.
4. Rutishauser U, Gall WE, Edelman GM: J Cell Biol 79:382–393, 1978.
5. Buskirk DR, Thiery JP, Rutishauser U, Edelman GM: Nature 285:488–489, 1980.
6. Bertolotti R, Rutishauser U, Edelman GM: Proc Natl Acad Sci USA 77:4831–4835, 1980.
7. Brackenbury R, Rutishauser U, Edelman GM: Proc Natl Acad Sci USA 78:387–391, 1981.
8. Orr CW, Roseman S: J Membr Biol 1:109–124, 1969.
9. Barbera AJ, Marchase RB, Roth S: Proc Natl Acad Sci USA 70:2482–2486, 1973.
10. Gottlieb DI, Rock K, Glaser L: Proc Natl Acad Sci USA 73:410–414, 1976.
11. Merrell R, Glaser L: Proc Natl Acad Sci USA 70:2794–2798, 1973.
12. McGuire EJ, Burdick CL: J Cell Biol 68:80–89, 1976.
13. Roth SA, Weston JA: Proc Natl Acad Sci USA 58:974–980, 1967.
14. Vogel Z, Daniels MP, Nirenberg M: Proc Natl Acad Sci USA 73:2370–2374, 1976.
15. Vogel Z, Nirenberg M: Proc Natl Acad Sci USA 73:1806–1810, 1976.
16. Lambiotte M, Vorbrodt A, Benedetti L: CR Acad Sci Paris 275D:2539–2542, 1972.
17. Takeichi M: J Cell Biol 75:464–474, 1977.
18. Urushihara H, Ozaki HS, Takeichi M: Dev Biol 70:206–216, 1979.
19. Takeichi M, Ozaki HS, Tokunaga K, Okada TS: Dev Biol 70:195–205, 1979.
20. Grunwald GB, Geller RL, Lilien J: J Cell Biol 85:766–776, 1980.