ADHESION AMONG NEURAL CELLS OF THE CHICK EMBRYO

III. Relationship of the Surface Molecule CAM to Cell Adhesion and the Development of Histotypic Patterns

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ABSTRACT

We have previously identified a molecule (named cell adhesion molecule [CAM]) that is involved in the in vitro aggregation of neural cells from chick embryos. In the present report, specific anti-CAM antibodies have been used to demonstrate that CAM is localized in neural tissues, and is associated with the plasma membrane of retinal cells and neurites. Furthermore, it has been shown by antibody absorption techniques that the decreased adhesiveness of cultured retinal cells obtained originally from older embryos is correlated with a decrease in the density or accessibility of cell adhesion molecules on the surface of these cells. The central role of CAM in neural cell aggregation has been established by the observation that anti-CAM Fab' fragments inhibit adhesion between neural cells in a variety of assays.

To investigate the function of CAM and cell adhesion in developing tissues, aggregates of retinal cells that are capable of forming histotypic patterns in vitro were cultured in the presence and absence of anti-CAM Fab'. The Fab' was found to inhibit sorting out of cell bodies and neurites and to decrease the number of membrane-membrane contacts, suggesting that CAM is associated with cell-cell, cell-neurite, and neurite-neurite interactions.

KEY WORDS cell-cell adhesion · antibodies · histotypic development · cell surface proteins · retinal cells · neurites

Cells obtained from retinal tissue by trypsinization will aggregate rapidly (3, 6, 18, 21), provided they have been allowed to recover from damage caused by the enzyme (3). Culture of the aggregates for a period of days then results in histotypic changes in cell position and morphology, a process that has been called sorting out (16, 25, 29-32).

Cell aggregation and sorting out have been studied extensively, the central rationale being Holtfreter's proposition that selective affinities among cells are a major factor in formation of tissue patterns during embryogenesis (10). A number of more detailed hypotheses have emerged subsequently (1, 5, 9, 16, 19, 20, 26), but neither the precise mechanism nor the physiological role of retinal cell aggregation is known.

We recently described the isolation of a cell adhesion molecule (CAM) from retinal tissue (3, 28). The conclusion that CAM is involved in cell-cell binding was based on its ability to neutralize the adhesion-blocking activity of monovalent Fab' fragments prepared from anti-retinal cell antibod-
ties as well as on the demonstration that specific anti-CAM Fab' fragments inhibit initial aggregation of retinal cells. The CAM molecule, which has an apparent mol wt in sodium dodecyl sulfate of \( \sim 140,000 \), can be labeled by iodination of intact cells using the lactoperoxidase procedure, implying that it is present on the cell surface (28).

Because of the indirect nature of the assay for CAM and the artificial conditions used in measuring cell adhesion in vitro, our subsequent studies have been concerned with providing further evidence that CAM is directly associated with cell-cell binding and with an analysis of the physiological role of cell adhesion in embryonic development. In the present report, we have used anti-CAM antibodies to determine the distribution of CAM on retinal cells, in developing retinal tissue, and throughout the chick embryo. The relationship between cell surface adhesion molecules and cell aggregation has been examined by antibody absorption techniques, and inhibition of cell-cell binding by anti-CAM has been used to assess the importance of CAM in adhesion phenomena reported by other laboratories. To investigate the physiology of cell adhesion, anti-CAM Fab' has been used to perturb the function of CAM during formation of histotypic aggregates of retinal cells.

MATERIALS AND METHODS

Antibodies, Fab' Fragments, and Lectins

The procedures for purification of CAM from chick embryo retina, production of anti-CAM and anti-retinal cell antibodies in rabbits, and preparation of monovalent Fab' fragments have been described in detail elsewhere (3, 28). Antibodies to chick embryo fibroblasts were obtained after 10 intraperitoneal injections at weekly intervals of rabbits with cells from secondary cultures. Concanavalin A and the divalent succinyl-concanavalin A derivative were prepared as reported previously (8).

Cells and Adhesion Assays

Suspensions of retinal cells were obtained by trypsinization (0.5% trypsin [Difco Laboratories, Detroit, Mich.] in calcium-free medium, 20 min, 37°C) of neural retina tissue from chick embryos. The tissue was washed three times to remove the enzyme and dispersed into single cells by trituration with a pipette. Over 95% of these cells were viable as judged by trypan blue exclusion. For adhesion assays and for absorption of antisera, retinal cells were allowed to recover from trypsinization by culturing them in 100-ml spinner flasks (Belloco Glass, Inc., Vineland, N.J.) containing Modified Eagle Medium with spinner salts (SMEM; Microbiological Associates, Walkersville, Md.) for 12-24 h at 400 rpm. Liver cells were obtained from 10-day embryos by treatment of the tissue with a mixture of trypsin, collagenase, and chick serum as described by Coon (4).

Cell adhesion was measured using three different assays: binding of cells in suspension to cells immobilized in a monolayer (24, 33), decrease in the number of single cells as measured by a particle counter (3, 18), and binding of radiolabeled membrane vesicles to cells (13). The procedure for preparation of vesicles (13) was modified by using a continuous gradient of sucrose (0.5-2 M), labeling with \(^{125}\)I using Chloramine T (34), and removing aggregates of vesicles by centrifugation for 3 min at 1,500 rpm in a clinical centrifuge. Membranes from \( 2 \times 10^6 \) cells were incubated with \( 5 \times 10^7 \) cells for 20 min at 37°C with continuous rotation (70 rpm), and unbound vesicles were removed by pelleting the cells through SMEM containing 4% bovine serum albumin.

Monolayer and Aggregate Cultures

To obtain monolayer cultures, \( 10^6 \) cells from 10-day embryos were suspended in 2 ml of Dulbecco's Modified Eagle Medium supplemented with 1/10 volume of fetal calf serum (DMEM; Microbiological Associates) and incubated at 37°C under 13% CO\(_2\) in 35-mm plastic petri dishes (BioQuest, BBL & Falcon Products, Cockeysville, Md.) for 2-3 days. For aggregate cultures (16, 31, 32), \( 10^7 \) cells were suspended in the same medium, placed in 35-mm plastic petri dishes, and rotated (70 rpm) on a gyratory shaker (Fisher Scientific Co., Pittsburgh, Pa.) for 7-8 days. The effect of Fab' fragments on development of histotypic patterns was examined by adding the antibody to the medium after initial formation of aggregates in culture over a 24-h period. All cultures were fed on alternate days by removing 1 ml of supernate and replacing it with fresh medium.

Absorption of Adhesion-Blocking Antibodies with Cells

We have previously described an assay for CAM based on the inhibition of adhesion by Fab' fragments prepared from antibodies against retinal cells, and neutralization of the Fab' by soluble antigens released from retinal tissue in culture (3). In the present experiments, the relative amount of cell adhesion molecules on the surface of cells was estimated in the same manner, except that the Fab' was neutralized by incubation with varying amounts of retinal cells from 8- or 14-day-old embryos for 20 min at 4°C and removal of the cells by centrifugation.

Localization of CAM in Aggregates, on Cells, and in Tissues

CAM was localized by use of specific anti-CAM antibodies in conjunction with either fluorescein-labeled goat anti-rabbit immunoglobulin (15) or sequential treatment with anti-immunoglobulin and a complex of rabbit...
anti-horseradish peroxidase and horseradish peroxidase, which can be visualized through an H₂O₂-dependent oxidation of diaminobenzidine (27). Cells in monolayers or small aggregates were treated directly with these reagents. Tissues or cells in large aggregates were first immersed in Tissue-Tek O.C.T. Compound (Lab-Tek Products, Div. Miles Laboratories Inc., Naperville, Ill.), frozen in liquid nitrogen, and cut into 10-μm sections using an International CTF microtome-cryostat. (Damon/IEC Div., Needham Heights, Mass.) The sections were fixed in 70% ethanol, washed in 50% ethanol, and equilibrated in saline, pH 7.4, before incubation with 25 μg/ml anti-CAM antibody. To minimize nonspecific staining in the peroxidase procedure, goat serum (20% vol/vol) was present in all solutions except the diaminobenzidine and peroxide. No staining was observed with either the peroxidase or fluorescence methods when immunoglobulin from unimmunized rabbits was used instead of anti-CAM.

**Electron Microscopy**

Cell aggregates were treated with glutaraldehyde, (2.5%, 20 min, 25°C) and osmium tetroxide (1%, 1 h, 25°C); dehydrated through 50, 70, 95, and 100% ethanol; embedded in Epon, sectioned on a Porter-Blum microtome, (DuPont Instruments-Sorvall, DuPont Co., Wilmington, Del.) and observed in a Philips 300 electron microscope. To detect CAM in cell micrographs, the peroxidase-anti-peroxidase procedure (see above) was introduced between the glutaraldehyde and osmium tetroxide fixations. To illustrate the histology of cell aggregates, representative fields were chosen from over 100 sections obtained from the same and different aggregates.

**RESULTS**

Previous studies have indicated that CAM is intimately involved in adhesion among chick embryo neural cells (3, 28). The experiments reported here were designed to demonstrate the presence of the molecule on the plasma membrane, to examine the relationship of cell adhesiveness to the relative density of CAM or related molecules on the cell surface, and to determine the distribution of CAM in embryonic tissues. Anti-CAM Fab' has been used as a specific probe to explore the role of adhesion in the formation of histotypic structures in cultured cell aggregates, and the relevance of CAM to adhesion studies carried out by other laboratories has been evaluated.

**Detection of CAM on Cells Obtained from Retinal Tissue**

If CAM is directly involved in cell adhesion, it should be present on the external surface of the plasma membrane. Furthermore, differences in the in vitro adhesiveness of retinal cells obtained from embryos of different ages (24) might be expected to reflect the amount or accessibility of such molecules on the cell surface. To examine the first point, retinal cells from 10-day-old embryos were aggregated for 20 min, and stained for cell surface CAM by use of anti-CAM antibody and the peroxidase-anti-peroxidase procedure. As shown in Fig. 1, CAM was present on most if not all areas of the cell surface including regions of close cell-cell contact. There was no indication that CAM was more concentrated in contact regions than on exposed areas of membrane. In addition, the pattern of anti-CAM staining on single or aggregated cells was similar, suggesting that adhesion did not induce visible changes in the distribution of CAM.

In previous studies, it has been shown that cultured retinal cells obtained by dissociation of tissues from 8-day-old embryos aggregate about four times faster than cells from 14-day embryos (24). To examine the possibility that the density of adhesion molecules accessible on the surface of retinal cells influences their rate of aggregation in vitro, the relative amount of these molecules on the two cell populations was estimated (3) by the ability of the cells to absorb out the adhesion-blocking activity of anti-retinal cell Fab'. It was found that about four times more 14-day-old cells were required to produce the same degree of absorption as 8-day cells (Fig. 2). Because the cells obtained from 8- and 14-day retinas are similar in size and surface area (our unpublished scanning electron microscope studies), this result raises the possibility that after trypsinization and culture the 8-day cells have about four times more Fab'-absorbing antigens exposed on their surface than the 14-day-old cells. Previous experiments demonstrated that neutralization of the adhesion-blocking activity of anti-retinal cell Fab' by antigens released from retinal tissue in culture is due to CAM or fragments of CAM. In the present experiments, the Fab' was reacted with a different source of antigens, the surface of intact cells, and therefore it is possible that the absorption assay detected cell adhesion molecules other than CAM. Adhesion between neural cells in vitro can be completely inhibited by anti-CAM, however, so it would appear that the cell surface antigens that absorbed the adhesion-blocking activity of anti-retinal cell Fab' were either CAM or a molecule associated with CAM function.

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Distribution of CAM in Embryonic Tissues and its Presence on Neuronal Processes

To examine the distribution of CAM more directly both within a tissue and throughout the embryo, frozen sections of tissue were stained using anti-CAM antibodies. With 6- to 14-day-old embryos, dense staining was observed in retina, brain, optic nerve, spinal cord, and both sympathetic and dorsal root ganglia. Muscle and liver cells were lightly stained, but it is not clear whether this represented the presence of a small amount of CAM, a minor contaminant in the antibody, or a nonspecific binding of the stain. Regional staining within a tissue was particularly dramatic in the retina, and exhibited a marked dependence on the stage of development (Fig. 3). On day 7, when the retina has not yet assumed its characteristic layered structure, CAM was found in similar amounts throughout the tissue. By day 14, when the retinal cell and neurite layers are clearly demarcated, the most intense staining was detected in the two plexiform regions.

The presence of CAM in plexiform layers suggests that it is associated with neuronal processes. To examine this possibility more closely, retinal cells were cultured for several days so that isolated neurite outgrowths could be observed. When fixed with glutaraldehyde and treated with anti-CAM antibody followed by fluorescein-labeled anti-immunoglobulin, neurite bundles were the most brightly stained, while thin processes and neural cell bodies displayed a similar level of fluorescence (Fig. 4). Fibroblastic cells that were also present in the cultures bound very little, if any, antibody. The anti-CAM stain on both cells and neurites appeared to be diffusely distributed within the resolution of the fluorescence technique.

Relevance of CAM to Other Studies on Cell Adhesion

We have shown previously (3, 28) that monovalent Fab' fragments prepared from specific anti-
FIGURE 2 Absorption of adhesion-blocking Fab' fragments by retinal cells from 8- and 14-day-old chick embryos. Absorption is expressed as the percent decrease in the amount of inhibition of aggregation of 10-day retinal cells produced by 1 mg of the Fab', and reflects the presence of adhesion molecules on the cell surface. 1 mg of the unabsorbed Fab' inhibited the rate of cell aggregation by 50%. The amount of cells used in each absorption is given in terms of packed cell volume, with 10 μl of pellet equal to 1 × 10^7 cells.

CAM antibodies inhibit binding between both retinal and brain cells (Table I). This observation is consistent with the present demonstration that CAM is found in many neural tissues. To examine the possibility that CAM is involved in cell adhesion phenomena previously described by other workers, Fab' was tested for its ability to inhibit both homologous and heterologous adhesion between cells from dorsal and ventral regions of the retina (2, 7), binding of membrane vesicles to retinal cells (13), and aggregation of liver cells (12, 22) (Table I). In all experiments with neural cells, initial adhesion was strongly inhibited by anti-CAM Fab'. In contrast, aggregation of liver cells was not affected by the antibody.

Effect of Anti-CAM Fab' on Histotypic Development of Cell Aggregates

The detection of CAM on nerve processes during the time when plexiform layers are being formed in the retina suggests that it might function in the development of these layers. We have not at present explored this hypothesis in vivo, but have carried out experiments in vitro on histotypic development of retinal cell aggregates, particularly in relation to the sorting out of cells and neurites. If 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 (31, 32). We examined the distribution of CAM in these aggregates (Fig. 5), and observed that, as in the retina, CAM was found primarily in neurite regions. When the aggregates that had been formed in culture over a 1-day period were transferred to medium containing 0.5–1 mg/ml anti-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. 6). 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'.

The effect of anti-CAM Fab' on differentiation of preformed retinal cell aggregates was also examined by electron microscopy (Fig. 7). These studies revealed that both cell bodies and neurites are densely packed after culture in medium with Fab' from unimmunized rabbits (Fig. 7a), but have a relatively loose arrangement in aggregates cultured with anti-CAM Fab' (Fig. 7b). As observed in the light microscope studies, the dense aggregates were clearly separated into neurite and cell body regions, whereas in the presence of anti-CAM the nuclei and processes were intermixed. At higher magnification, (Fig. 7c–d), it appeared that the number of membrane-membrane contacts between neurites was decreased in the presence of anti-CAM. The two types of aggregates were not obviously different with respect to cell classes, intracellular morphology, and total number of cells or neurites per aggregate.

DISCUSSION

The major conclusions of this work are that the CAM molecule exists on the plasma membrane of retinal cells and their processes, that two cell populations which differ in their rates of aggregation appear to have a different relative density or accessibility of cell adhesion molecules on their surfaces, and that the formation of large, segregated cell body and neurite regions in aggregates is inhibited by anti-CAM Fab'. The following discussion will first consider some technical aspects of the studies on the distribution of CAM, and the relationship of this work on cell
adhesion to previous work. It will then consider the mechanism and significance of the effect of anti-CAM on formation of retinal tissue and some implications of these studies for analysis of the development of the nervous system in general.

On initial consideration, the studies on the distribution of CAM in retina would suggest that CAM is preferentially located on neuronal processes. This result, however, is subject to an important qualification. The plexiform layers of neural tissue are composed almost entirely of fine neurites, and therefore contain more surface membrane per unit volume than tissues containing cell bodies. Consequently, the intense staining of these layers does not necessarily mean that CAM is more concentrated on neurites than cell bodies. In fact, when observed in culture by immunofluorescence microscopy, the amount and distribution of CAM on membranes of neural cell bodies and processes appeared quite similar; the absence of CAM on fibroblastic cells, however, was consistent with the observation that there is less if any CAM in non-neural tissue.

The correlation between the rate of aggregation in vitro of 8- and 14-day retinal cells and the density or accessibility of adhesion molecules on the cell surface supports the hypothesis that CAM and other molecules associated with CAM function are intimately involved in the formation of cell-cell bonds. While such observations are useful in defining the chemistry of cell adhesion, their implications for developmental control in vivo remains to be determined. Although the cells used in the in vitro assay had been obtained by dissociation of tissue and had been allowed to re-express surface proteins, it cannot be assumed...
FIGURE 4 Presence of CAM on neurite outgrowth from cultured retinal cells: (a) phase contrast; (b) fluorescence microscopy after staining with anti-CAM immunoglobulin and fluorescein-labeled anti-immunoglobulin. × 650.

| Binding between:* | Assay used | Fab' from unimmunized rabbits | Anti-CAM Fab' |
|-------------------|------------|-------------------------------|---------------|
| Cell A            | Cell B     |                               |               |
| 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 membranovesicles | Centrifugation | 8.2               | 1.4           |
| Liver             | Liver      | Particle counter             | 31            | 33            |

* Brain, retinal and liver cells from 6-, 8-, and 10-day-old embryos, respectively.
† 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.
that they were identical in surface composition to cells in tissues. In fact, from the amount of reaction product of the peroxidase labeling procedure seen in frozen sections of intact tissues, it appeared that a 14-day retina has at least as much CAM as an 8-day retina. This suggests that the low concentration of CAM on cells obtained from the 14-day tissue reflects a decrease in ability to produce the molecule in culture after trypsinization.

Research on aggregation of chick embryo cells has involved several quite different experimental approaches, and it has proved difficult to formulate a consistent interpretation of the results obtained. In attempting to relate our work to that described in the literature, we have re-examined the fundamental adhesion phenomena of several other studies, particularly those which measure initial binding events. These include the binding of membrane vesicles to cells (13), dif-

FIGURE 5 Distribution of CAM in frozen sections of retinal cell aggregates as revealed by fluorescence microscopy after staining with anti-CAM immunoglobulin and fluorescein-labeled anti-immunoglobulin. (a) Phase contrast; (b) fluorescence microscopy. Neurite and cell body regions that appeared in these aggregates during culture for 8 days are indicated by N and C, respectively. × 240.

FIGURE 6 Typical histology of retinal cell aggregates after culture for 8 days: (a) aggregate after culture in medium containing 1 mg/ml Fab' from unimmunized rabbits. Distinct neurite (N) and cell body (C) regions have formed. (b) Aggregate cultured in medium containing 1 mg/ml anti-CAM Fab'. Sorting out into neurite and cell body regions has not occurred. Samples are Epon sections stained with toluidine blue. × 330.
FIGURE 7  Electron micrographs of retinal cell aggregates (Fig. 6) after culture for 8 days in medium containing 1 mg/ml Fab' from unimmunized rabbits (a and c), or 1 mg/ml anti-CAM Fab' (b and d). In Fig. 7a, the cells and neurites have sorted out into densely packed regions with numerous membrane-membrane contacts including neurite-neurite interactions (c). In Fig. 7b, the cells are loosely arranged and intermixed with neurites. Little neurite-neurite contact (d) was observed. These histologies were typical of sections obtained from different regions of the same aggregate, and of sections from different aggregates cultured under the same conditions. (a and b) × 5700; (c and d) × 30,000.

ferences in adhesion among cells obtained from the dorsal and ventral regions of the retina (2, 7), and variations in binding between retinal, liver, and brain cells (12, 21, 22). In most cases we were able to reproduce the reported phenomena, and in all cases binding of neural cells was strongly inhibited by anti-CAM. It would therefore appear that the same binding event is being examined in most studies on neural cell adhesion, and that this process involves the cell surface molecule CAM.

In contrast, aggregation of liver cells in vitro,
which can be inhibited by antibodies against these cells (our unpublished results), was unaffected by anti-CAM antibody. This observation suggests that different molecules are involved in liver cell adhesion and is consistent with reports that liver cells bind poorly to neural cells (12, 21, 22). Furthermore, in preliminary studies we have found that extracts of liver membranes do not neutralize the adhesion-blocking properties of anti-CAM. On the other hand, our results are not consistent with previous reports that adhesion between retinal and brain cells is tissue-specific (1, 16). Instead, they support the hypothesis that the mechanism of cell adhesion in vitro is basically the same for most neural cells (24).

A variety of molecules found in retinal tissue has been reported to influence cell adhesion (1, 9, 14, 17, 19, 20). The available information (molecular weights, tissue specificity, effects on aggregation) does not suggest that they are related to CAM. Although it is not as yet possible to conclude whether these molecules are complementary to CAM in their function, their relevance to cell adhesion may be clarified once it has been demonstrated directly that CAM is a ligand and to what cell surface structures it binds.

In any case, given that blockade of CAM function by anti-CAM Fab' prevents cell-cell binding, it is possible to use this reagent to probe the role of cell adhesion during development. Ideally, this analysis would involve intact embryos, but our initial experiments have been carried out on cell aggregates because of the ease with which the cells can be exposed to milligram quantities of antibody over a period of several days. The effect of anti-CAM on formation of histotypic aggregates suggests that cell-cell, cell-neurite, and neurite-neurite interactions are necessary for the separation of cell bodies and neurites into discrete regions, but not for viability of the cells and growth of the neurites. It will be of interest to determine whether synapse formation, most of which occurs in retinal tissue or aggregates after appearance of neurite regions (11, 31, 32) is prevented by anti-CAM Fab' or whether it is entirely independent of CAM-mediated adhesion and the sorting out of cells and processes.

The significance of the present observations for the specificity of cell interactions is simultaneously obscured and heightened by the fact that CAM can be detected in essentially all nerve tissues and on all parts of the nerve cell membrane, including the cell bodies, neurite shafts and growth cones (our unpublished observation). Despite its wide distribution, specificity in the function of CAM could occur as a result of quantitative changes (25, 26) in the cell surface density of CAM as a function of time or position (24), a similar change in a molecule that interacts with CAM, or as a yet undetected heterogeneity in CAM structure. In studies on cultured spinal ganglia, we have observed that anti-CAM Fab' affects the morphology, but not the extent of neurite outgrowth, apparently by preventing side-to-side adhesions that gather individual processes into bundles (23). These results are consistent with the changes in histotypic aggregates reported here and support the hypothesis that adhesive functions associated with CAM are a property of nervous system tissues in general.

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