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

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Robert Brackenbury, Urs Rutishauser, and Gerald M. Edelman*

The Rockefeller University, 1230 York Avenue, New York, New York 10021

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ABSTRACT Three criteria have been used to distinguish among different systems of embryonic cell adhesion: dependence on Ca++, 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++. Aggregation of cells prepared from retina or brain without Ca++ did not require Ca++ 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++ did require Ca++ 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++ 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++-independent system displayed a limited tissue specificity, mediating binding to neural but not liver cells. In contrast, the Ca++-dependent systems of both neural and liver cells caused binding to all cell types tested. The Ca++-dependent system was most active in retinal cells from 6-7 day embryos, whereas the Ca++-independent system was most active at later times during development. In addition, treatments that inhibited the Ca++-independent or Ca++-dependent systems had very different effects on the fasciulation of neurites from dorsal root ganglia. All of the results suggest that Ca++-independent and Ca++-dependent adhesion systems play different functional roles during organogenesis.

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 (1.25% GIBCO) in Ca++-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++/Mg++-free medium (14) supplemented with 10 mM CaCl2; and (iii) "T/E," limited trypsinization (with 0.002% twice-recrystallized trypsin for 15 min at 37°C in Hepes-buffered, Ca++/Mg++-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 x 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++/Mg++-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/ Ca++) and specific antibodies (anti-N-CAM, anti-L-CAM, and anti-T/Ca cell antibodies) were assayed by enzyme-linked immunosorbent assay (ELISA) (16). The results are shown in Table 1.
Ca cell that inhibit Ca\(^{2+}\)-dependent adhesion of retinal cells were produced by intramuscular, intraperitoneal, and subcutaneous injections of rabbits with 5 \(\times\) 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\(^\prime\) 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). Hepses-buffered, Ca\(^{2+}/Mg\(^{2+}\)-free medium was prepared with either 10 mM CaCl\(_2\) or 1 mM EDTA added to 5 \(\times\) 10\(^6\) neural cells or 2 \(\times\) 10\(^6\) liver cells to a final volume of 2 ml. In some experiments, the cells were preincubated (15 min at 4\(^\circ\)C) in 0.2 ml of Fab\(^\prime\) (5 mg/ml). Cell suspensions were shaken (90 rpm; 37\(^\circ\)C for neural cells, room temperature for liver cells) in scintillation vials. Aliquots removed at 0, 20, and 40 min were centrifuged and cell pellets were counted using a Cytoflor (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 Hepses-buffered medium containing CaCl\(_2\). These cells (2 ml) were labeled internally by adding 0.5 ml of fluorescein diacetate (10 \(\mu\)g/ml) and incubating for 10 min at room temperature. The cells were washed twice and resuspended to 1 \(\times\) 10\(^6\) 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 \(\mu\)g/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\(^\prime\) 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 \(\times\)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 mg 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.

Because antibodies against N-CAM blocked only the calcium-dependent 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).

# Table 1. Aggregation of retinal cells prepared by different techniques

<table>
<thead>
<tr>
<th>Preparation*</th>
<th>Aggregation properties</th>
<th>% inhibition by Fab(^\prime)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ca(^{2+}), T/SC: 0.5% trypsin and culture</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>in suspension</td>
<td>+</td>
</tr>
<tr>
<td>T/E: 0.002% trypsin and with 1 mM EDTA</td>
<td>-</td>
<td>60</td>
</tr>
<tr>
<td>T/Ca: 0.04% trypsin and with 10 mM Ca(^{2+})</td>
<td>-</td>
<td>6</td>
</tr>
</tbody>
</table>

*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.

† Hepses-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\(^\circ\)C.

Ca\(^{2+}\)-dependent and Ca\(^{2+}\)-independent aggregation systems of 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...
Cells in an apparently partially independent, adhesion system, appear to have Ca\textsuperscript{2+}-dependent binding (Table I), which was obtained with 10 mM CaCl\textsubscript{2} for T/Ca and C/T/B cells, but not inhibited by anti-(N-CAM) or anti-(L-CAM) antibodies. These results suggest that both the L-CAM and neural Ca\textsuperscript{2+}-dependent systems participate in the binding. Consequently, each of these cell types appears to have components capable of interacting with the Ca\textsuperscript{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 I). When the monolayer was composed of T/E cells, the binding of T/E cells was Ca\textsuperscript{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\textsuperscript{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.


eare different. To indicate the time during development at which Ca\textsuperscript{2+}-dependent and Ca\textsuperscript{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\textsuperscript{2+}-dependent aggre-

### Table 2. Aggregation of cells from different tissues

<table>
<thead>
<tr>
<th>Cell preparation</th>
<th>Tissue</th>
<th>Aggregation rate</th>
<th>Anti-(N-CAM)</th>
<th>Anti-(L-CAM)</th>
<th>Anti-T/Ca cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>T/E</td>
<td>Retina</td>
<td>66</td>
<td>94</td>
<td>8</td>
<td>18</td>
</tr>
<tr>
<td>T/E</td>
<td>Brain</td>
<td>58</td>
<td>81</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>T/Ca</td>
<td>Retina</td>
<td>54</td>
<td>9</td>
<td>0</td>
<td>68</td>
</tr>
<tr>
<td>T/Ca</td>
<td>Brain</td>
<td>53</td>
<td>6</td>
<td>0</td>
<td>57</td>
</tr>
<tr>
<td>C/T/B\textsuperscript{t}</td>
<td>Liver</td>
<td>35</td>
<td>9</td>
<td>61</td>
<td>26</td>
</tr>
</tbody>
</table>

* See Table 1.
† Expresssed 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\textsuperscript{2+}/Mg\textsuperscript{2+}-free medium with 1 mM EDTA (for T/E cells) or 10 mM CaCl\textsubscript{2} for T/Ca and C/T/B cells) was used.
‡ Treatment with a mixture of collagenase, trypsin, and bovine serum (11, 19).

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\textsuperscript{2+}-dependence and pattern of inhibition by antibodies.

The binding between liver cells and neural T/E cells was Ca\textsuperscript{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\textsuperscript{2+}-independent system.

The binding of liver cells to neural T/Ca cells was Ca\textsuperscript{2+}-dependent, partially inhibited by either anti-(L-CAM) or anti-T/Ca cell antibody alone and strongly inhibited when both anti-

### Table 3. Binding between cells having different adhesion systems

<table>
<thead>
<tr>
<th>Heterotypic binding between</th>
<th>Cell in monolayer</th>
<th>Cell in suspension</th>
<th>Ca\textsuperscript{2+}, 10 mM</th>
<th>Number of cells bound to monolayer*</th>
<th>Anti-(N-CAM)</th>
<th>Anti-(L-CAM)</th>
<th>Anti-T/Ca cell</th>
<th>Anti-LCAM plus anti-T/Ca cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver C/T/B\textsuperscript{t}</td>
<td>Neural T/E</td>
<td>+</td>
<td>202</td>
<td>0</td>
<td>63</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver C/T/B\textsuperscript{t}</td>
<td>Neural T/Ca</td>
<td>+</td>
<td>402</td>
<td>39</td>
<td>32</td>
<td>73</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neural T/Ca</td>
<td>Neural T/E</td>
<td>+</td>
<td>216</td>
<td>0</td>
<td>75</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neural T/Ca</td>
<td>Neural T/Ca</td>
<td>+</td>
<td>266</td>
<td>80</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

* Twenty microscope fields representing a total area of 1 mm\textsuperscript{2} were scored.
† In cases where Ca\textsuperscript{2+}-dependent binding was obtained, the percentages shown were calculated after subtraction of the binding obtained in the absence of Ca\textsuperscript{2+}.
‡ Cells treated with a mixture of collagenase, trypsin, and bovine serum (11, 19).
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 6-7-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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