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A monoclonal antibody disrupting calcium-dependent cell–cell adhesion of brain tissues: Possible role of its target antigen in animal pattern formation

(adhesive specificity/tissue segregation/animal morphogenesis)

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ABSTRACT **The Ca^{2+}-dependent cell–cell adhesion system (CDS) is thought to be essential for the formation and maintenance of cell adhesion in a wide variety of tissues. Previous studies suggested that CDS has some cell-type specificity; for example, the monoclonal antibody ECCD-1 selectively recognizes CDS of certain epithelial tissues in mouse embryos but not nervous tissues. In the present study, we have obtained a monoclonal antibody, designated NCD-1, that disrupts connections between brain cells of mouse embryos. A series of experiments suggested that NCD-1 specifically recognizes CDS. We then determined the distribution of the NCD-1 antigen in various mouse tissues. NCD-1 reacted with cells of the following tissues and cell lines: nervous tissues from various sources, lens, striated muscle, cardiac muscle, glioma G26-20, adrenocortical tumor Y1, and melanoma B16. None of these cells reacted with ECCD-1, and the cells reactive with ECCD-1 did not react with NCD-1. There was also a class of cells that did not react with either ECCD-1 or NCD-1. These results suggest that cells in the body can be classified into at least three groups containing CDS of differing specificities. A map of the tissue localization of these different classes of CDS also suggests that the expression of cell-type-specific cell adhesion molecules in each tissue plays a crucial role in adhesion between the same cell types and segregation of different cell types in processes essential for animal morphogenesis.**

Recent studies have succeeded in identifying several kinds of cell–cell adhesion molecules implicated in construction of vertebrate tissues. Molecules belonging to the Ca^{2+}-dependent cell–cell adhesion system (CDS) are thought to be particularly important in initiation and maintenance of cell adhesion in various tissues, since inhibition of the CDS results in disruption of cell–cell adhesion, affecting embryonic morphogenesis (1–3). While CDS is present in a wide variety of tissues, an interesting property of CDS is its cell-type-specific function (4). We have previously shown that when two different types of cells such as teratocarcinoma and fibroblastic cells are combined, they cannot cross-adhere to one another by CDS, suggesting that the CDS in these two cell types is distinct in specificity (5). This hypothesis was later confirmed with the monoclonal antibody ECCD-1, which was raised against the CDS of teratocarcinoma cells (6).

Studies on the binding of ECCD-1 to various cells of mouse embryos showed that its target molecules are detected in all the cells of early embryo (2, 6). However, during development they are lost from many tissues such as connective tissues and brain, although they persist in a number of types of epithelial cell in various organs (6). Thiery *et al.* (7) performed a detailed analysis in chicken embryos of the tissue distribution of liver cell adhesion molecule (L-CAM), a molecule equivalent to the antigen of ECCD-1 (8). They found that it is present in the ectoderm of early embryos but disappears from mesodermal cells at the stage of gastrulation and from nervous tissues at the stage of invagination of the neural plate. Thus, disappearence of L-CAM seems to be correlated with certain events in morphogenesis (9).

In the present series of studies, we attempt to characterize the CDS in cells that do not react with ECCD-1. For this purpose, we obtained a monoclonal antibody termed NCD-1 that reacts with CDS in brain cells, and then we analyzed the tissue distribution of its antigens. The results show that the antigens of ECCD-1 and NCD-1 are expressed in completely different cell populations. On the basis of this tissue distribution pattern of CDS, we discuss a general scheme of involvement of this adhesion system in morphogenetic processes in animal development.

MATERIALS AND METHODS

Cell Lines. Teratocarcinoma AT805 (5), adrenocortical tumor Y1 (10), neuroblastoma Neuro 2a (11), melanoma B16 (12), and PSA5-E (13) were cultured in a 1:1 mixture of Dulbecco's modified Eagle's minimal essential medium and Ham's F-12 medium supplemented with 10% fetal calf serum (DH10F). Glioma G26-20 (14) was cultured in Ham's F-12 medium with 10% fetal calf serum. Myeloma P3-X63-Ag8-U1 (15) and hybridoma were also maintained in DH10F.

Primary Cell Cultures. Various organs were isolated from fetal ICR mice (Shizuoka Laboratory Animal Center) of the following ages post coitus: whole brain of 10.5 days; otic vesicle of 10.5 days; lens and epidermis of 11.5 days; dorsal root ganglia and heart of 12 days; spinal cord, lung, and liver of 13 days; skeletal muscle, salivary gland, neurohypophysis, and adenohypophysis of 18 days. Pancreas, stomach, and thyroid were obtained from newborn ICR mice. Mammary gland and endothelium of common carotid artery were isolated from adult ICR mice. Adult lens and skeletal muscle were also used.

These tissues were dissociated by treatment with trypsin, collagenase, or both and cultured with DH10F in 3.5-cm-diameter Falcon dishes coated with collagen. These cultures were usually prepared 1 or 2 days before use. Clonal cultures of skeletal muscles were prepared by T. Kagawa (Toneyama National Hospital) as described previously (16) and used after 9 days in culture.

Preparation of Hybridomas and Monoclonal Antibodies. A rat (Wistar strain, Shizuoka Laboratory Animal Center) was immunized by an intraperitoneal injection of whole brain tissues obtained from 30 ICR mouse fetuses of 9.5 days post

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Abbreviations: CDS, Ca^{2+}-dependent cell–cell adhesion system; CIDS, Ca^{2+}-independent cell–cell adhesion system; L-CAM, liver cell adhesion molecule; CI, cytotoxicity index.

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coitus. These tissues were homogenized during injection by passage through the injection needle. After three weeks, a booster injection of the same antigens was performed three times at 11-day intervals, and, 3 days after the last injection, spleen cells of this animal were fused with mouse myeloma P3-X63-Ag-U1 cells. For screening hybridomas, we added their culture fluids to monolayer cultures of brain cells obtained from mouse fetuses of 10.5 days post coitus, and we examined for induction of morphological changes in brain cell colonies by microscopy.

For production of antibodies, hybridomas were either cultured in serum-free medium as described by Murakami *et al.* (17) or grown in nude rats, from which ascites fluid and serum were collected. These animal fluids were heated at 56°C for 30 min to inactivate complement. When necessary, antibodies were purified from the serum-free culture medium by precipitation with 50%-saturated ammonium sulfate and gel filtration through a Sephacryl S300 column.

Dissociation and Aggregation of Cells. Cells were dissociated by treatment with three kinds of trypsin solutions, 0.01% trypsin (type I, Sigma) plus 1 mM $CaCl_2$ (TC), 0.01% trypsin plus 1 mM EGTA (TE), or 0.0001% trypsin plus 1 mM EGTA (LTE) as described (5, 18), and aggregation of these dissociated cells was assayed with a Coulter Counter as described (5, 18). The extent of aggregation was represented by the index N_t/N_0 described previously (18). Inhibition of cell aggregation by antibodies was calculated by the formula given in our previous paper (18).

Complement-Dependent Cytotoxicity Assay. Complement (Low-Tox-M rabbit complement; Cedarlane Laboratories, Hornby, ON) reconstituted with distilled water was diluted to 10% with a 1:1 mixture of Dulbecco's modified Eagle's minimal essential medium and Ham's F-12 medium containing 0.3% bovine serum albumin buffered with 25 mM Hepes to pH 7.4 (H-DHM). This solution was incubated with 10% (wt/vol) acetone powder of adult brain tissues in an ice bath for 1 hr to remove nonspecific cytotoxic activity and used immediately after clarifying by centrifugation. The acetone powder was prepared by homogenizing brains in acetone and drying under reduced pressure.

For cytotoxicity tests using suspended cells, 10^6 cells were first incubated in 1 ml of rat hybridoma ascites fluid diluted 1 to 100–200 with H-DHM at 4°C for 60 min. After removal of this solution by centrifugation, cells were resuspended in 1 ml of the above absorbed complement solution and incubated at 37°C for 60 min. The cells were sedimented and incubated with 0.1% trypan blue in Hanks' solution for 5 min in an ice bath for determination of percent lysed cells.

When monolayer cultures were used for this assay, we incubated cells with 1 ml per 3.5-cm dish of rat hybridoma ascites fluid diluted 1 to 100–200 with the above complement solution at 37°C. After appropriate incubation (usually 4 hr maximum), the nonlysed cells were counted in randomly selected areas of culture dishes under a phase microscope and compared to the original cell number in the same area to calculate viability. As a control, heat-inactivated nonimmunized rat serum was added to the solutions instead of hybridoma ascites fluid.

The degree of cytotoxicity was represented by the cytotoxicity index (CI), defined as 100 × [(percent lysed cells in the presence of NCD-1 − percent lysed cells in the absence of NCD-1)/(100 − percent lysed cells in the absence of NCD-1)].

RESULTS

A Monoclonal Antibody, NCD-1, Inhibiting Brain CDS. We obtained one hybridoma clone that secreted antibody, termed NCD-1, with the following biological effect on brain cell cultures. Brain cells obtained from 10.5-day embryos formed compact clusters in which cells were densely packed when cultured overnight (Fig. 1 *a* and *c*). When the culture medium contained NCD-1, the brain cells did not form compact clusters but instead formed clusters with looser cell–cell connections (Fig. 1 *b* and *d*).

Because these observations suggested that NCD-1 affects cell–cell adhesion, we more quantitatively analyzed the effect of this antibody on cell–cell adhesion. It has been shown that cells of the nervous system as well as of other tissues contain two distinct cell–cell adhesion systems, CDS and a Ca^{2+}-independent system (CIDS) (4, 18). Treatment of cells with 0.01% trypsin in the presence of Ca^{2+} (TC treatment) leaves only CDS intact, while treatment with a low concentration (0.0001%) of trypsin in the absence of Ca^{2+} or in the presence of EGTA (LTE treatment) leaves only CIDS intact. Treatment of cells with 0.01% trypsin in the presence of EGTA (TE treatment) inactivates both adhesion systems.

We examined whether NCD-1 inhibits aggregation of brain cells dissociated by any of the above trypsin treatments. We found that aggregation of TC-treated cells was inhibited by this antibody in a concentration-dependent manner but that of LTE-treated cells was not (Fig. 2). ECCD-1 did not affect aggregation of brain cells.

The NCD-1 antibody belongs to the IgM class as judged by its molecular weight, and it displayed complement-dependent cytotoxicity for brain cells. When brain cells were subjected to the complement-dependent cytotoxicity test after different trypsin treatments, TC-treated cells were killed by NCD-1, but neither LTE-treated nor TE-treated cells were cytolyzed (Table 1). All of these results suggest that NCD-1 specifically binds to components of CDS.

Effect of NCD-1 on a Glioma Cell Line. Cells of a glioma line G26-20 also reacted with NCD-1. As found with brain cells, aggregation of TC-treated G26-20 cells was inhibited by NCD-1 but that of LTE-treated cells was not (Fig. 2). Complement-dependent cytotoxicity tests also showed that this antibody lyses TC-treated cells but not LTE- or TE-treated cells (Table 1). These results further indicated that the target of NCD-1 is CDS. ECCD-1 showed no effect on aggregation of G26-20 cells.

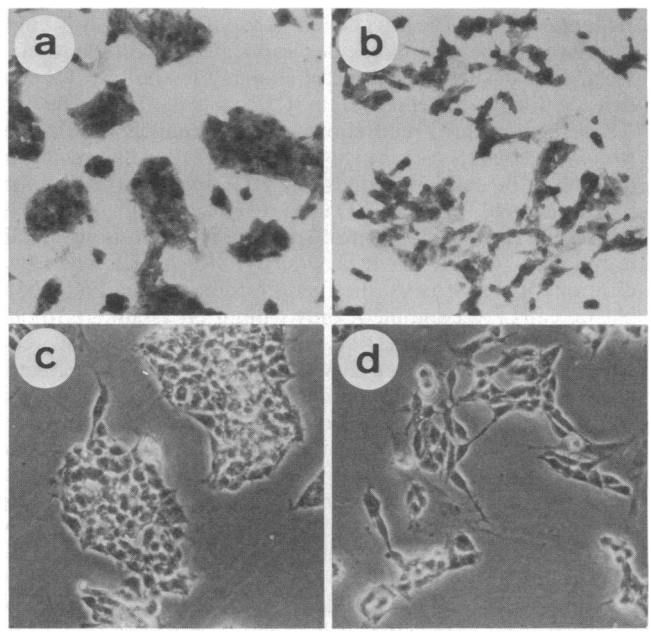

FIG. 1. Effect of NCD-1 on the colony formation of brain cells. Cells freshly prepared from 10.5-day fetus were cultured for 16 hr in the absence (*a* and *c*) or presence (*b* and *d*) of NCD-1 (10 μg/ml). (*a* and *b*, Giemsa staining, ×300; *c* and *d*, phase contrast, ×600.)

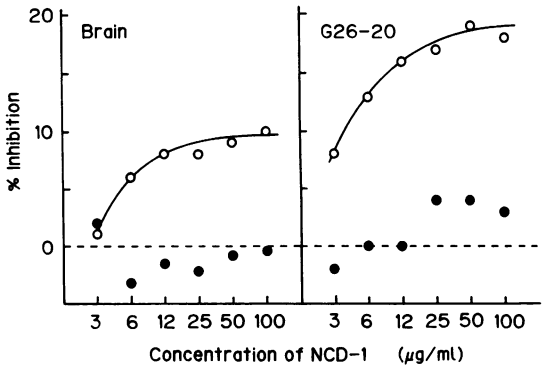

FIG. 2. Effect of NCD-1 on the aggregation of brain and G26-20 cells dissociated by TC treatment (○) or LTE treatment (●). Brain cells obtained from 10.5-day fetus were cultured for 1 day before use. Aggregation was measured in the medium containing 1 mM $CaCl_2$ after 20 min of incubation. Percent inhibition of aggregation was calculated by the formula given in ref. 18. Each value represents the mean of triplicate determinations.

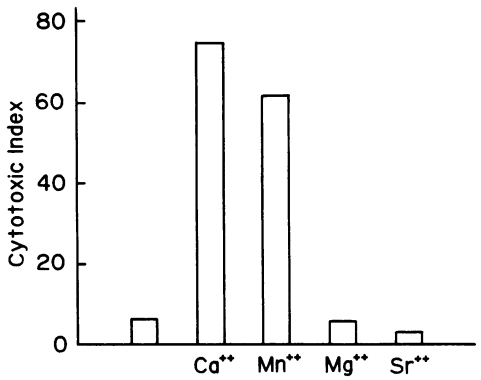

FIG. 3. Effect of divalent cations on the binding of NCD-1 to surfaces of G26-20 cells. For this experiment, TC-treated cells were first incubated with NCD-1 at 6 μg/ml in Ca^{2+}- and Mg^{2+}-free saline supplemented with various divalent cations (chloride salts) at 4°C for 60 min and washed briefly with the same solution without the antibody. The cells were then incubated with the standard complement solution and their viability was assayed.

Our previous study showed that the monoclonal antibody ECCD-1, which recognizes CDS in teratocarcinoma cells, binds to its target molecules in a Ca^{2+}-dependent manner. We thus tested NCD-1 for divalent cation dependence in its binding to cell surfaces by means of the complement-dependent cytolysis assay. The results showed that NCD-1 binds to cell surfaces only in the presence of Ca^{2+} or Mn^{2+} (Fig. 3).

Cell-Type Specificity of NCD-1 Targets. We examined which cell types in mouse tissues are reactive with NCD-1, comparing them to the ECCD-1-sensitive cell types. For detecting NCD-1-sensitive cells we employed a complement-dependent cytotoxicity test because this was found to be the most sensitive method for the present purpose. To identify ECCD-1-sensitive cells, we assayed the adhesion-blocking effect of this antibody (6) by adding it to monolayer cultures of cells. (Immunohistochemical staining was not sufficiently sensitive to detect binding of these antibodies, and ECCD-1 showed no cytotoxic effects.)

Primary cultures of cells obtained from various organs of fetal and adult mice were prepared. In cultures of brain cells obtained from 10.5-day fetuses in which multiple cell types were detected, most cells reacted with NCD-1 after incubation with complement for 4 hr (CI > 90), although cells with fine processes, probably neurons, tended to require longer incubation periods to be killed. Similar results were obtained with dorsal root ganglia (Fig. 4 *a* and *b*) and spinal cord. In these experiments, none of the cells in control plates, which contain complement and nonimmune rat serum, were significantly lysed during incubation, indicating that the observed cytotoxic effect was due to specific binding of NCD-1 to its antigen. In summary, all cells tested from nervous tissues appear to have antigens identified by NCD-1, although their amount per cell might vary with cell types.

NCD-1 sensitivities of cells in other organs and lines were determined by a similar assay. Cell lines were tested after TC treatment, because they generally became more sensitive to NCD-1 after this treatment. Cells of the following tissues and lines were found to be sensitive to NCD-1, as judged by high value of CI (>90): lens epithelium, neurohypophysis, cardiac muscle, myotubes of skeletal muscle, adrenocortical tumor Y1, and melanoma B16. The majority of mononucleated myoblasts in skeletal muscle were resistant to NCD-1 (CI = 37), as found with a clonal culture of these cells (Fig. 4 *c* and *d*). Fibroblastic cells present in muscle tissues also did not react with NCD-1. None of the NCD-1-sensitive cells was found to react with ECCD-1. Aggregation of lens epithelial, Y1, and B16 cells dissociated by TC treatment was inhibited by NCD-1 but not by ECCD-1 (data not shown).

Table 1. Complement-dependent cytotoxic effect of NCD-1 on cells dissociated by different trypsin treatments

Cells	Treatment	% lysed cells* With NCD-1	% lysed cells* Without NCD-1	CI
Brain	TC	97	23	96
	LTE	32	36	−6
	TE	30	29	1
G26-20	TC	95	36	92
	LTE	11	14	−3
	TE	37	40	−5

*Approximately 200 cells were counted to obtain each value. In the absence of NCD-1, serum of nonimmunized rats was present.

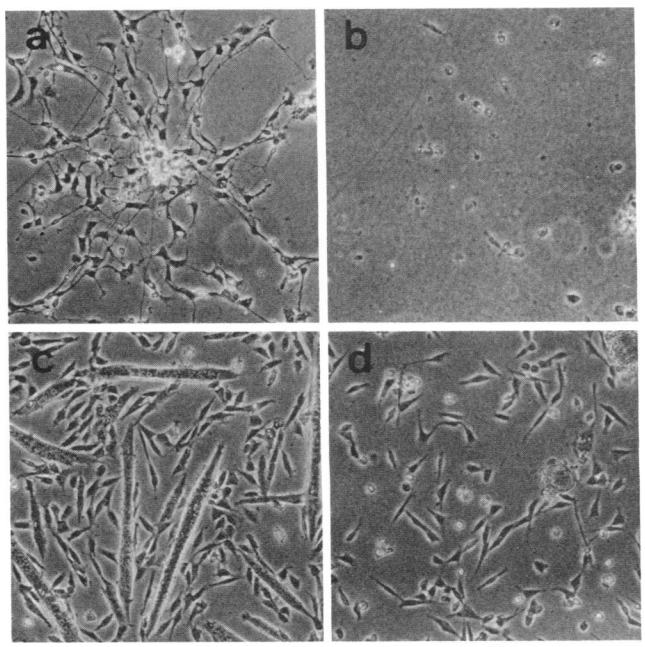

FIG. 4. Complement-dependent cytotoxic effect of NCD-1 on cells of various tissues cultured. (*a* and *b*) Dorsal root ganglion; (*c* and *d*) adult skeletal muscle in clonal culture. (×300.) (*Left*) Before addition of NCD-1 and complement; (*Right*) the same fields as in *Left* 4 hr after addition of NCD-1 and complement.

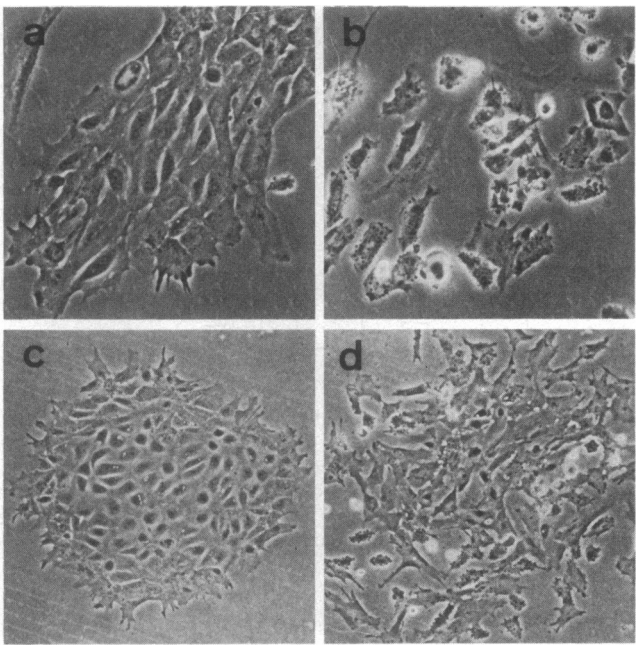

FIG. 5. Effect of ECCD-1 on cell–cell adhesion of salivary gland epithelium (*a* and *b*) and otic vesicle epithelium (*c* and *d*). Ascites fluid containing ECCD-1 was added to cultures of these tissues and incubated (see ref. 6 for method). (*Left*) Before addition of the antibody; (*Right*) the same field as in *Left* 4 hr after addition of the antibody. (*a* and *b*, ×600; *c* and *d*, ×300).

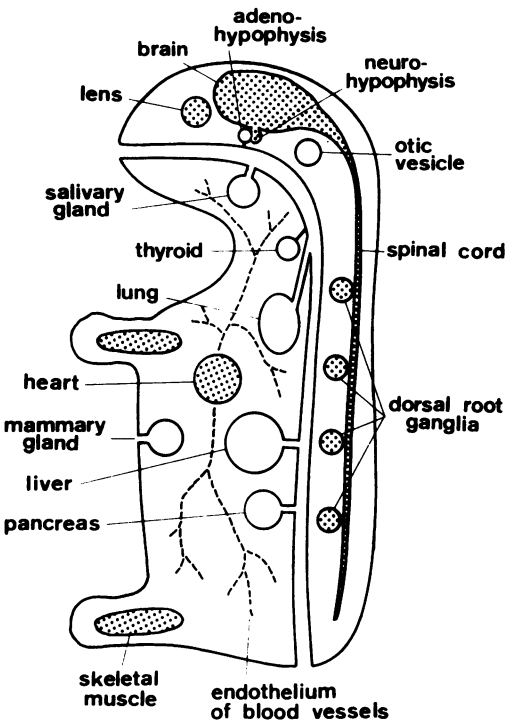

FIG. 6. Distribution of tissues containing cells reacting with ECCD-1 or NCD-1. Areas drawn only by a solid line represent tissues reactive with ECCD-1; stippled areas, tissues reactive with NCD-1; broken line, tissues reactive with neither ECCD-1 nor NCD-1. This figure does not include all the cells tested in the present experiment.

(Other NCD-1-sensitive cells were not tested in this aggregation assay.)

Epithelial cells of the following tissues did not react with NCD-1, but their cell–cell adhesion was disrupted by ECCD-1: epidermis, mammary gland, liver, pancreas, stomach, salivary gland (Fig. 5 *a* and *b*), thyroid, and adenohypophysis. Epithelial components of otic vesicle reacted with ECCD-1 (Fig. 5 *c* and *d*), but some nonepithelial cells present in cultures of this tissue also reacted with NCD-1. (It was not determined whether these NCD-1-sensitive cells were genuine components of otic vesicle or derived from surrounding tissues.) One-cell and eight-cell stage (compacted) embryos and teratocarcinoma AT805 cells, which were previously shown to be sensitive to ECCD-1 (6), were resistant to NCD-1. Aggregation of these ECCD-1-sensitive cells dissociated by TC treatment was not inhibited by NCD-1 (data not shown).

Fibroblastic cells present in cultures of the above ECCD-1-sensitive tissues generally did not react with either ECCD-1 (see also ref. 6) or NCD-1. Endothelial cells of artery, neuroblastoma Neuro 2a cells, and PSA5-E cells, which were identified as visceral endoderm cells, were also resistant to both ECCD-1 and NCD-1.

DISCUSSION

CDS is present in a wide variety of cell types, probably in all kinds of cells that form solid tissues. Both NCD-1 and ECCD-1 antibodies inhibited cell–cell adhesion mediated by CDS of various tissues, but these two kinds of antibodies recognized distinct cell types, as schematically summarized in Fig. 6. Some groups of cells, such as fibroblasts of various tissues and endothelial cells of blood vessels, did not react with either NCD-1 or ECCD-1. These results suggest that CDS should be divided into subclasses with different immunological specificity: an NCD-1-reactive type, an ECCD-1-reactive type, and a third, nonreactive, type. These subclasses of CDS are probably also distinct in functional specificity, that is, they may not be cross-reactive to each other in an aggregation assay, as has been shown by the lack of cell–cell adhesion in a combination of teratocarcinoma CDS and fibroblast CDS (5).

The antigens identified by NCD-1 and ECCD-1 showed similar Ca^{2+} sensitivity; they are both protected by Ca^{2+} against proteolysis and require Ca^{2+} not only for expressing their activity but also for reacting to each antibody. Mn^{2+} was also effective in inducing binding of both of these antibodies to cell surfaces as found in the previous (6) and present experiments. These suggest that they have some common molecular structure. The target of ECCD-1 with such properties has already been identified (6); the major component of this target was a protein with a molecular weight of 124,000, termed "cadherin." Similar molecules were also identified by other groups (19–22). So far the target of NCD-1 has not been identified because NCD-1 does not form stable antigen–antibody complexes in immunoprecipitation or immunoblot experiments. Grunwald *et al.* (23) have described a cell surface protein of neural retina cells with a molecular weight of 130,000 that is protected by Ca^{2+} against proteolysis, as a candidate for a component of the CDS of this tissue. It is possible that this 130,000 molecular weight protein is related to a target of NCD-1, since both of them are implicated in CDS of nervous tissues.

Assuming that the targets of NCD-1 and ECCD-1 are related to each other (since both are involved in CDS), we call the hypothetical antigens of NCD-1 "N-cadherin" and the identified antigens of ECCD-1 "E-cadherin," thus functionally subdividing the term "cadherin" originally given to the latter antigens.

Is N-cadherin related to previously identified cell adhesion molecules? So far, at least three types of cell–cell adhesion molecules have been detected from nervous tissue by using monoclonal antibodies: N-CAM (and its related molecules) (24), Ng-CAM (25), and L1 antigen (26), all of which were reported to be involved in CIDS. Since CDS is a func-

tional system completely independent of CIDS (27–29), N-cadherin should be distinct from any of these CIDS molecules.

It should be noted, however, that the observed distribution of N-cadherin is surprisingly similar to that of N-CAM as reported by Thiery *et al.* (30), when compared in embryos at stages after the completion of neural induction, although there are exceptions [for example, Neuro 2a cells have N-CAM (31) but do not express N-cadherin]. It seems, therefore, that the expression of these two distinct kinds of specific cell adhesion molecules may be under some shared regulatory mechanism. Such a coordinate expression of two kinds of cell-type-specific adhesion molecules would provide each cell with greater adhesive specificity.

The next question is how the multiple classes of CDS participate in the morphogenesis of embryos. An important histological feature of the observed distribution pattern of CDS is that cells with different subclasses of CDS generally do not form direct contact with each other. For example, epithelial cells with E-cadherin are always separated from fibroblastic cells without E-cadherin by a barrier such as a basement membrane. Thus, a general principle may be that cells form direct contacts only with cells having a homotypic subclass of CDS, at least *in vivo*. We also experimentally demonstrated that, when two cell types expressing different subclasses of CDS are brought together, they spontaneously segregated from each other (5). Therefore, if some fraction of cells in a cell population expressing only one type of CDS express a new subclass of CDS, they would automatically segregate from the original population.

Our previous study showed that all embryonic cells express E-cadherin during early cleavage (2, 6). Cells at this developmental stage, however, did not react with NCD-1, indicating that expression of N-cadherin begins at a later stage. We also previously showed that early embryos do not express a fibroblast type of CDS (32). Therefore, while all embryonic cells originally express E-cadherin, cells in many parts of the body cease expression of this antigen at some developmental stage and instead initiate expression of a new class of cadherin molecules, such as N-cadherin.

For instance, in neural tube formation, this tissue loses L-CAM, a molecule equivalent to E-cadherin, during the process of invagination as shown by Thiery *et al.* (7). Similarly, in eye formation, the lens loses L-CAM during invagination. It is, therefore, possible that *de novo* expression of N-cadherin may occur in these tissues at the invagination stage following disappearance of E-cadherin. The epidermis (ectoderm) from which the neural tube and lens originally arose continues to express L-CAM or E-cadherin. Thus, expression of new specific adhesion molecules in invaginating tissues may be an important factor for their separation from overlying ectoderm. To verify such a hypothetical mechanism of tissue separation, we must accumulate more information on the on–off of expression of various subclasses of cadherin molecules.

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