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Two Chick Embryonic Adhesion Systems: Molecular vs Tissue Specificity

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We have investigated the adhesive properties of cells from several neural and nonneural chick embryonic tissues dissociated using modifications of the standard dissociation procedures employed routinely in this laboratory to obtain retinal cells. Each of these tissues (7-day optic tectum, retina, and heart, and 3.75-day limb bud) displayed both Ca^{++}-dependent (CD) and Ca^{++}-independent (CI) aggregation, the relative rates of which differed from tissue to tissue. In every case, cells prepared so as to display one mode of aggregation or the other cross-adhered readily to cells—regardless of tissue origin—displaying the same mode of aggregation. Cross adhesion was negligible between cells—even from the same tissue—prepared so as to display different modes of aggregation. Anti-retinal Fab molecules which inhibit selectively either the CI or CD aggregation of retina cells strongly inhibited the corresponding aggregation of optic tectum cells, but had no effect upon the aggregation (CI or CD) of heart cells. These results demonstrate the existence in the tissues examined of dual adhesion mechanisms similar in Ca^{++} dependence and recognition properties to those of the retina, but showing certain immunological distinctions from the latter. The immunological relationship among the adhesion mechanisms from the various tissues is under continuing investigation.

Key words: limb bud, Fab inhibition, Ca^{++} dependence, specificity, trypsin sensitivity, immunological analysis, cell surface antigen, cerebellum, chick embryo, retina, heart

Embryonic cells from different tissues, when artificially combined in mixed aggregates, generally sort out into separate regions according to their tissues of origin [1–9]. It has also been found that cells often initiate adhesions more rapidly to other cells from the same tissue than to cells from a different tissue [10–19]. These observations have led many investigators to seek tissue-specific "aggregation factors" responsible for cellular "recognition." Indeed, several such "factors" have been described [20–24]. More recently, it has been demonstrated that dissociated embryonic chick neural retina cells [25–30] and a variety of tissue culture cell lines [25, 26, 31–34] possess two distinct, functionally independent adhesion mechanisms—one Ca^{++} dependent, the other Ca^{++} independent—which are responsible for most of the aggregation behavior displayed by these cells. The different sensitivities of the two adhesion mecha-

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nisms to Ca^{++} and trypsin permit the removal of one, the other, or both adhesion mechanisms (Table I), and it has been demonstrated that two cells from a given tissue cross-adhere only if both possess at least one of these two mechanisms in an active state [25, 34, 35]. The discovery of such dual adhesion mechanisms raises the question of their role in generating selectivity of adhesion among cells from different embryonic tissues. A study of this question in our laboratory indicates that functionally—and in some cases immunologically—related dual adhesion mechanisms are present in many neural and non-neural tissues throughout the early chick embryo. Here we first present a sampling of data to illustrate these findings and then explore their implications in the matter of tissue-specific cell recognition factors.

MATERIALS AND METHODS

Media

Hanks' balanced salt solution supplemented with 1.5 mM Hepes buffer (HBSS) was used for all routine procedures. HBSS containing 2.5 mM Ca^{++} (2× Ca^{++} HBSS) was used in the preparation of TC cells, and Ca^{++}-free HBSS containing 1.3 mM EGTA (EGTA·HBSS) was used for steps requiring Ca^{++}-free conditions.

Cell Preparations

Retina E, LTC, LTE, TC, and TE cells (Table I) were prepared from 7-day White Leghorn chick embryos as described previously [29]. Similar preparations from optic tectum were obtained by substituting one tectal lobe for one retina in each dissociation procedure. Limb bud cells were prepared in a similar manner but with the following modifications. Fore and hind limb buds were dissected from 3.75-day (Hamilton stage 22) embryos. They were incubated for 15 minutes at 37°C in a solution containing 150 National Formulary Units (NFU) of trypsin (Miles, 3× recrystallized) per limb bud (TE or TC cells); 2.5 NFU of trypsin per limb bud (LTE and LTC cells); or no trypsin at all (E cells). The incubation solutions were prepared either with 2× Ca^{++} HBSS

TABLE I. A Summary of the Preparative Procedures for and the Adhesion Mechanisms Present on the Various Retinal Cell Preparations Described in the Text

Cell preparation	Dissociation medium			Adhesion medium	
	Trypsin per retina (NFU)	Ca^{++} (in mM)	EGTA (in mM)	CI	CD
E	—	—	1.3	+	(+)
LTC	68	2.5	—	+	+
LTE	68	—	1.3	+	—
TC	3000	2.5	—	—	+
TE	3000	—	1.3	—	—

Dissociation in EGTA, E; dissociation in trypsin plus EGTA, TE; dissociation in trypsin plus Ca^{++}, TC; dissociation in low trypsin plus EGTA, LTE; dissociation in low trypsin plus Ca^{++}, LTC; Ca^{++}-independent mechanism, CI; Ca^{++}-dependent mechanism, CD; present and active, +; present but undetected in the self-aggregation assay, (+); absent, —.

(LTC and TC cells) or with EGTA·HBSS (E, LTE, and TE cells). Typically, 160 limb buds were treated in 10 ml of incubation solution. Heart cells also were prepared using modifications of the procedures used for retina. Heart ventricles were dissected from 7-day embryos and the atria and epicardium were removed. The ventricles were gently massaged to remove blood, minced, and incubated for 15 min at 37°C either in a solution containing 3000 NFU of trypsin per ventricle (TE and TC cells) or in a solution containing no trypsin at all (E cells). EGTA·HBSS was used in the preparation of E and TE cells, and $2 \times Ca^{++}$ HBSS was used in preparing TC cells. Typically 40 ventricles were treated in 13.3 ml of incubation solution. For a fourth preparation of heart cells (C cells), ventricles were incubated for 15 min at 37°C in HBSS containing 1% collagenase (Worthington, type III), 1% BSA, and 200 Kalikrein units (KU) of aprotinin per ml.

All cell preparations were diluted into either HBSS or EGTA·HBSS containing 0.01% DNase I (Sigma).

Fluorescent-Labeled Cells

Variously prepared cells from the tissues listed above were labelled with fluorescein isothiocyanate (FITC) as described previously [35].

Aggregation Assays

All kinetic assays, including aggregation-inhibition experiments using Fab, were performed using 5×10^6 cells per ml in our particle aggregometer as described previously [29, 30, 36]. Results are plotted with aggregation represented by an increase in Δ mV. Cross-adhesion assays were performed at 37°C on a gyratory shaker at 60 rpm in 24-well plastic plates (Linbro) [35]. Except where indicated, retina and optic tectum were used at 2.5×10^6 cells per ml; limb bud and heart were used at 3.75×10^6 cells per ml. Results were obtained by parallel phase contrast and epifluorescence microscopic observation.

Antibody Preparations

The Fab fragments capable of selectively inhibiting either the Ca^{++}-dependent aggregation of retinal TC cells (R-15 Fab) or the Ca^{++}-independent aggregation of retinal E cells (R-9 Fab) are described elsewhere [30].

RESULTS

Optic Tectum

Dissociation of 7-day chick optic tecta using the five sets of conditions established for the dissociation of 7-day neural retina yielded tectal cell preparations which displayed aggregation properties indistinguishable in nearly every respect from those of similarly prepared retinal cells. In aggregation-inhibition experiments using anti-retina Fabs, optic tectum E cell (CI) or TC cell (CD) aggregation was inhibited only by the Fab from antibodies raised against the corresponding retina cell preparation (Fig. 1). The inhibition titer for each Fab was similar for the two tissues. Cross-adhesion experiments using FITC-labelled and unlabelled optic tectum cells prepared according to the five protocols had shown that, like retina cells [35], optic tectum cells cross-adhere

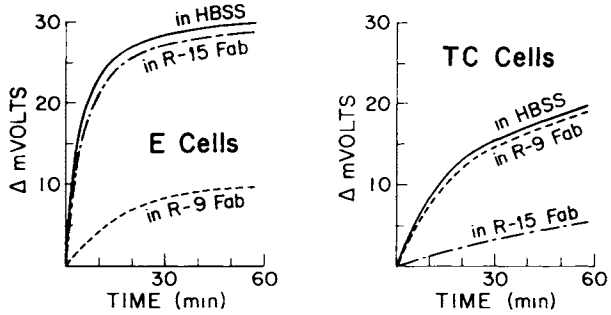


Fig. 1. Inhibition of optic tectum cell aggregation by anti-retina Fabs at 3 mg/ml. Left) Optic tectum E cells in HBSS (——), in anti-retina E cell (R-9) Fab (----), in anti-retina TC cell (R-15) Fab (-·-·). Right) Optic tectum TC cells in HBSS (——), in anti-retina E cell (R-9) Fab (----), in anti-retina TC cell (R-15) Fab (-·-·).

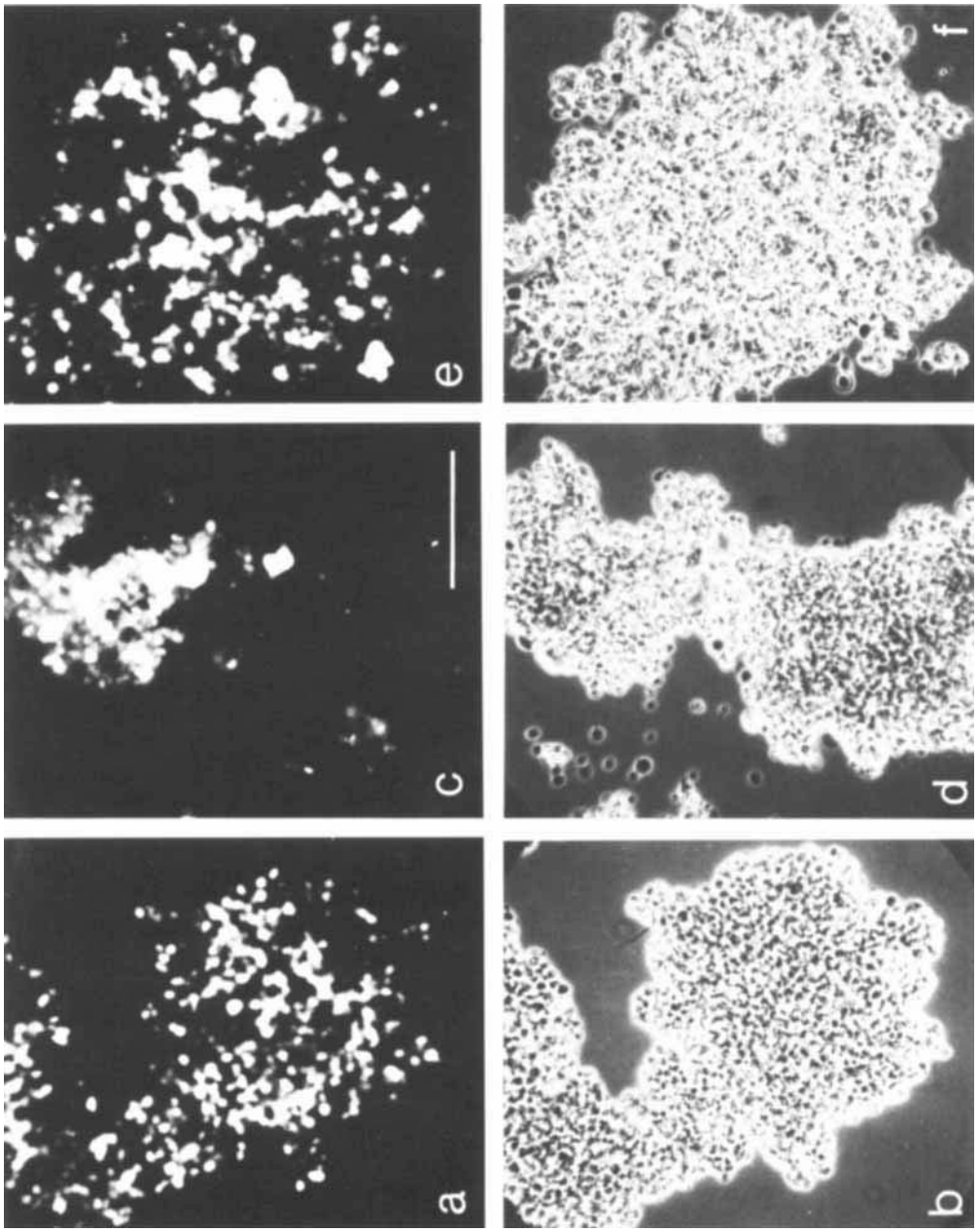
only if they share at least one active adhesion mechanism. When similar experiments were performed combining optic tectum and retina cells, it was again found that cells from the various preparations of each tissue would cross-adhere extensively only when they shared an adhesion mechanism (Fig. 2). Thus the dual adhesion mechanisms of the optic tectum are very similar to those of the retina in their physiological, immunological, and recognition properties.

Heart

Studies performed using 7-day heart gave results significantly different from those described above. Heart cells were dissociated using modifications of the five standard procedures developed for retina. Their aggregation was predominantly Ca^{++}-dependent, Ca^{++}-independent aggregation being negligible in all cases. Heart TC cells cross-adhered extensively with retina TC cells (CD mechanism only) but failed to cross-adhere significantly with retina LTE cells (CI mechanism only; Fig. 3). Neither the anti-retina E cell (R-9) nor the anti-retina TC cell (R-15) Fab had any inhibitory effect upon the self-aggregation of heart TC cells (Fig. 4). However, anti-retina TC cell Fab did prevent the incorporation of retina TC cells into heart TC cell aggregates (Fig. 5). These results demonstrate the existence in heart of a CD adhesion mechanism sharing biochemical (physiological) and functional characteristics with the CD mechanism of retina. However, no immunological relationship has been found. The data also suggest that retina TC cells employ the same CD mechanism for cross-adhesion to heart as for self-adhesion.

We found that heart cells can in fact display significant CI aggregation. Heart cells dissociated using 1% collagenase in HBSS ("C cells") instead of

Fig. 2. Paired fluorescence and phase contrast photographs of aggregates formed by coaggregation of variously prepared retina and optic tectum cells. a,b) FITC-labelled optic tectum LTE cells co-aggregate with unlabelled retina LTE cells in EGTA·HBSS. c,d) FITC-labelled optic tectum TC cells initially aggregate separately from unlabelled retina LTE cells in HBSS. e,f) FITC-labelled optic tectum TC cells co-aggregate with unlabelled retina TC cells in HBSS. Each pair of photographs shows a single microscopic field which represents accurately the distribution of labelled and unlabelled cells in each of several hundred observed aggregates. Bar = 100 μm.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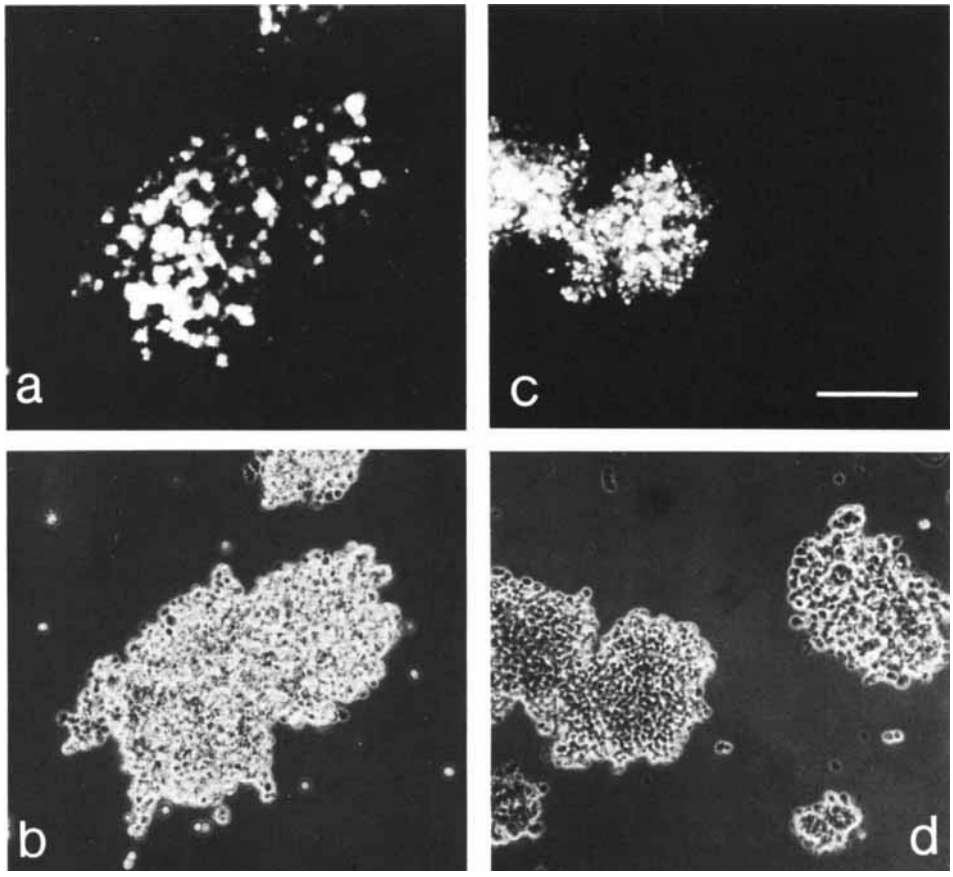

Fig. 3. Paired fluorescence and phase contrast photographs of aggregates formed by coaggregation of retina and heart cells. a,b) FITC-labelled retina TC cells co-aggregate with unlabelled heart TC cells in HBSS. c,d) FITC-labelled retina LTE cells aggregate separately from unlabelled heart TC cells in HBSS. Each pair of photographs shows a single microscopic field which represents accurately the distribution of labelled and unlabelled cells in each of several hundred observed aggregates. Bar = 100 μm.

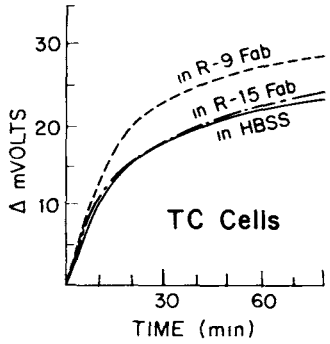

Fig. 4. Aggregation of heart TC cells in HBSS (——), in anti-retina E cell (R-9) Fab at 3 mg/ml (----), and in anti-retina TC cell (R-15) Fab at 3 mg/ml (·---·). Neither Fab is inhibitory.

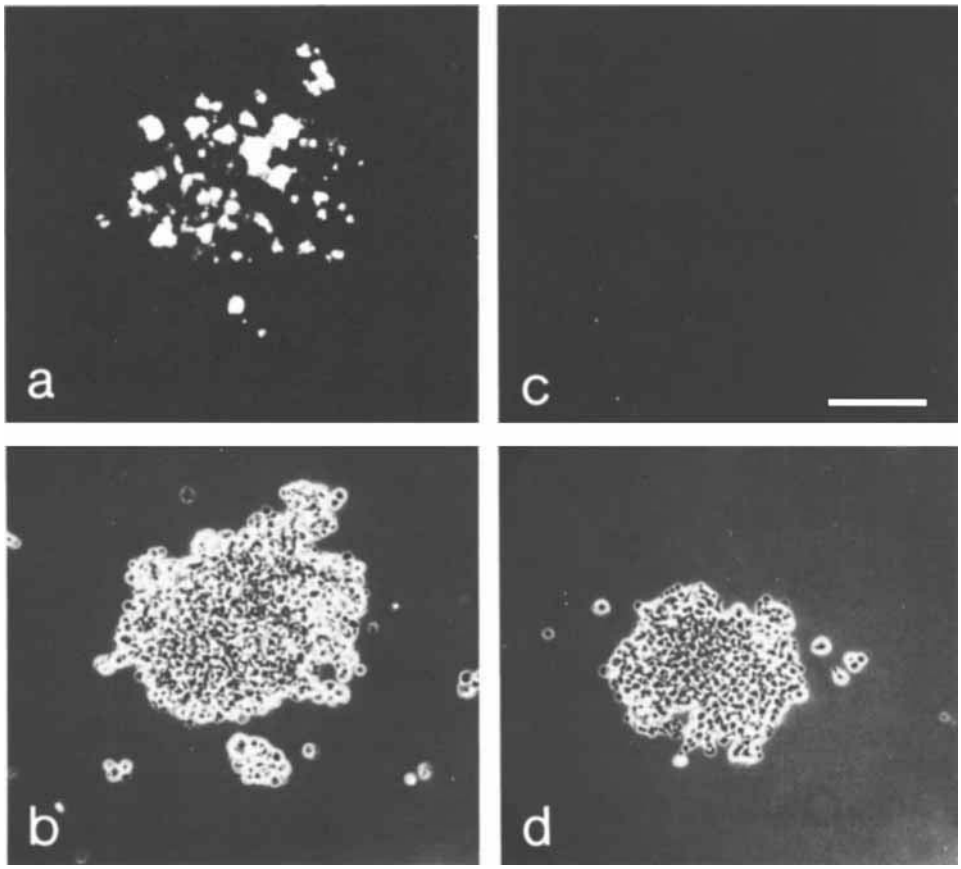

Fig. 5. Paired fluorescence and phase contrast photographs of FITC-labelled retina TC cells and unlabelled heart TC cells allowed to coaggregate in HBSS with (c,d) or without (a,b) anti-retina TC cell (R-15) Fab at 3 mg/ml. Retina and heart cells were at 1.25×10^6 and 5×10^6 cells/ml, respectively. This Fab inhibits the Ca^{++}-dependent cross-adhesion. Each pair of photographs shows a single microscopic field which represents accurately the distribution of labelled and unlabelled cells in each of several hundred observed aggregates. Bar = 100 μm.

trypsin displayed both CD and CI aggregation (Fig. 6). Neither mode of aggregation was inhibited by either anti-retina Fab, but heart C cells did cross-adhere with retina LTE cells (CI mechanism only) in the absence of Ca^{++} (Fig. 7). Furthermore, this cross adhesion was blocked by anti-retina E cell (R-9) Fab (Fig. 8). These experiments show that heart possesses a CI mechanism functionally related to the CI mechanism of retina, but once more no immunological relationship has been found. Again, the data suggest that retina cells employ the same CI mechanism for cross-adhesion to heart as for self-adhesion.

Limb Bud

Limb buds (3.75 days old) were dissociated using modifications of the five standard retinal dissociation procedures. These limb bud cells displayed both Ca^{++}-dependent and Ca^{++}-independent aggregation, but the CI aggregation was

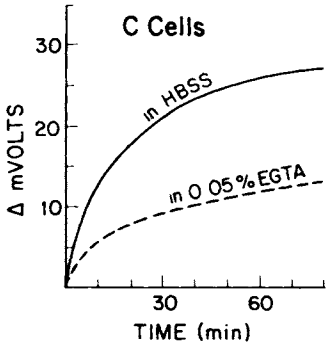

Fig. 6. Aggregation of heart C cells in HBSS (——) and in EGTA·HBSS (----).

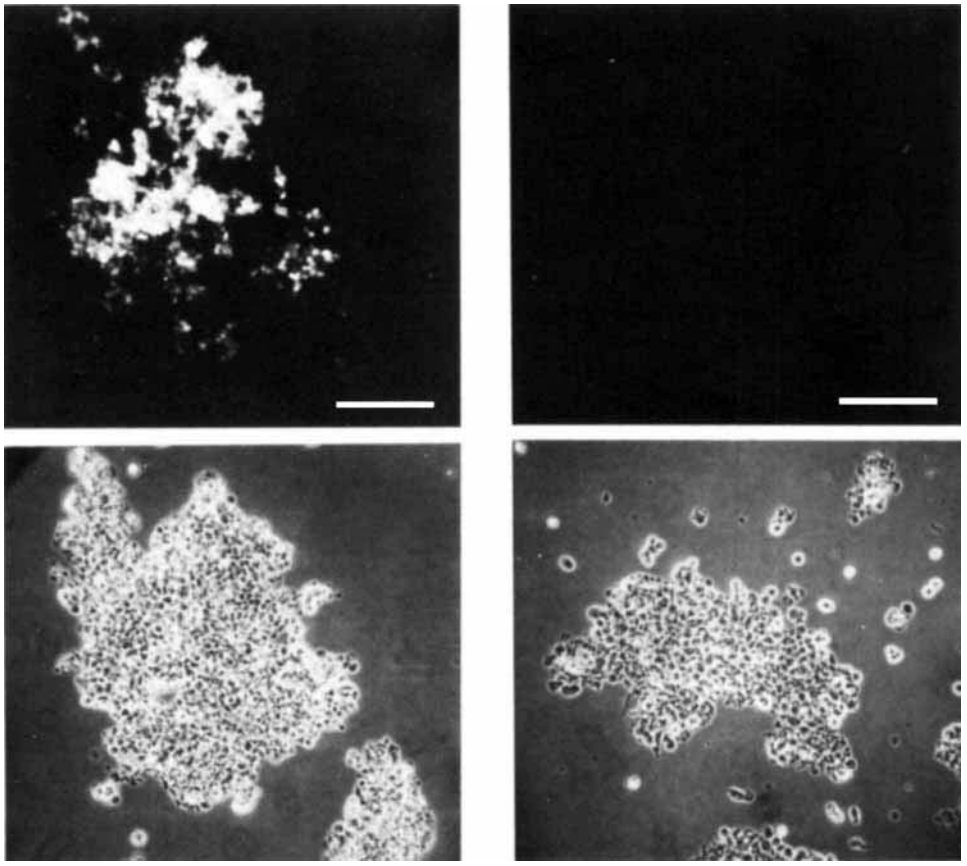

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Fig. 7. Paired fluorescence and phase contrast photographs of FITC-labelled retina LTE cells and unlabelled heart C cells allowed to coaggregate in EGTA·HBSS. The two kinds of cells cross-adhere. Retina and heart cells were at 1.25×10^6 and 5×10^6 cells/ml, respectively. This pair of photographs shows a single microscopic field which represents accurately the distribution of labelled and unlabelled cells in each of several hundred observed aggregates. Bar = 100μm.

Fig. 8. Paired fluorescence and phase contrast photographs of FITC-labelled retina LTE cells and unlabelled heart C cells allowed to co-aggregate in EGTA·HBSS with anti-retina E cell (R-9) Fab at 3 mg/ml. Compare with Figure 7. This Fab inhibits the Ca^{++}-independent cross-adhesion. Retina and heart cells were at 1.25×10^6 and 5×10^6 cells/ml, respectively. This pair of photographs shows a single microscopic field which represents accurately the distribution of labelled and unlabelled cells in each of several hundred observed aggregates. Bar = 100 μm.

less extensive and the separation of the two modes of aggregation by proteolytic dissection was less complete than with retina. Limb bud TC cells and retina TC cells formed completely interspersed aggregates (Fig. 9), as did limb bud LTE and retina LTE cells coaggregating in the absence of Ca^{++} (Fig. 10). By contrast, retina LTE (CI mechanism only) and limb bud TC (CD mechanism only) cells initially failed to cross-adhere significantly. Aggregates of the two kinds subsequently did cross-adhere, probably due to the restoration of the proteolytically removed adhesion mechanisms (Fig. 11). Thus limb bud cells also possess CI and CD adhesion mechanisms which share certain biochemical

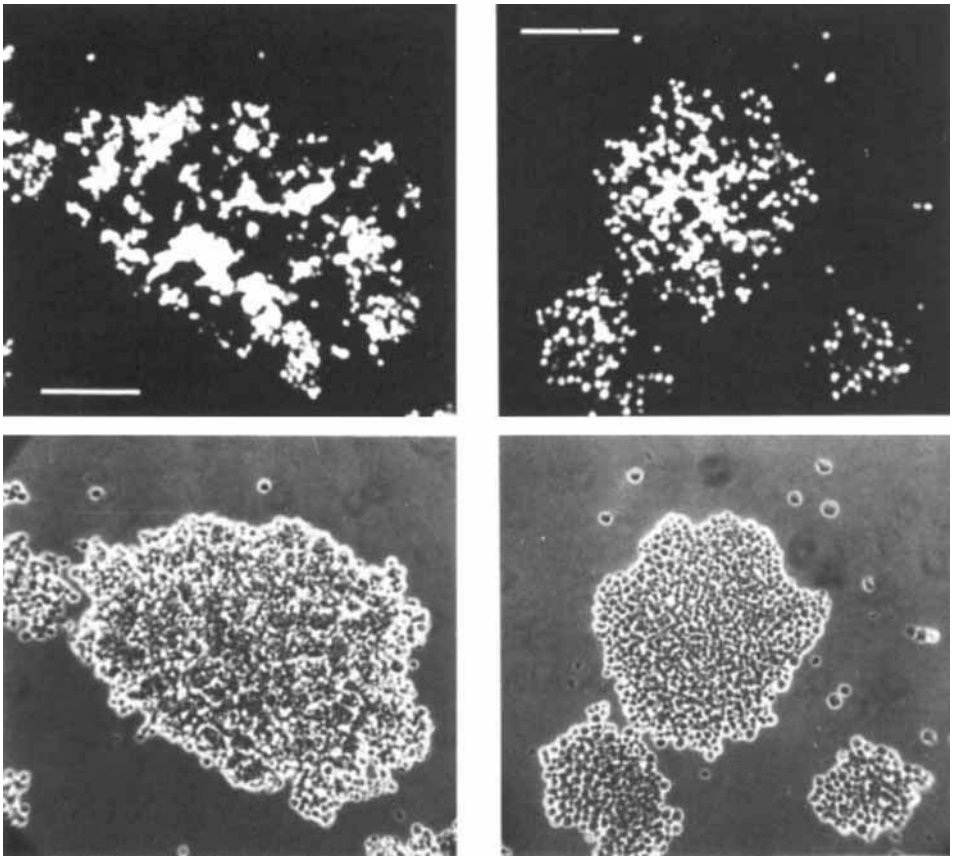

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Fig. 9. Paired fluorescence and phase contrast photographs of FITC-labelled limb bud TC cells and unlabelled retina TC cells allowed to co-aggregate in HBSS. They cross-adhere. This pair of photographs shows a single microscopic field which represents accurately the distribution of labelled and unlabelled cells in each of several hundred observed aggregates. Bar = 100 μm.

Fig. 10. Paired fluorescence and phase contrast photographs of FITC-labelled limb bud LTE cells and unlabelled retina LTE cells allowed to co-aggregate in EGTA•HBSS. They cross-adhere. This pair of photographs shows a single microscopic field which represents accurately the distribution of labelled and unlabelled cells in each of several hundred observed aggregates. Bar = 100 μm.

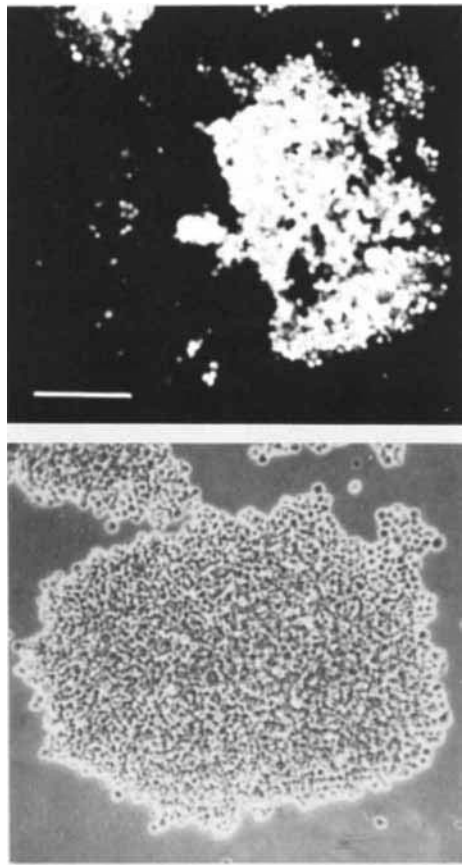

Fig 11 Paired fluorescence and phase contrast photographs of FITC-labelled limb bud TC cells and unlabelled retina LTE cells allowed to co-aggregate in HBSS The two kinds of cells initially formed separate aggregates which later fused This pair of photographs shows a single microscopic field which represents accurately the distribution of labelled and unlabelled cells in each of several hundred aggregates Bar = 100 μm

(physiological) and functional characteristics with the dual mechanisms of retina. The immunological relationship between the adhesion mechanisms of limb bud and retina is under investigation.

DISCUSSION

The data presented here indicate that dual adhesion mechanisms similar in ion dependence and recognition properties to those of retina are expressed also in other embryonic tissues, both neural and non-neural. Ca^{++}-dependent (CD) and Ca^{++}-independent (CI) aggregation both are manifest in the three em-

bryonic tissues reported upon here (optic tectum, heart, and limb bud), and cells from each tissue prepared so as to display only one of the two modes of aggregation cross-adhered appreciably to retina cells only if the latter displayed the same mode of aggregation. This apparent functional similarity among the corresponding adhesion mechanisms of the various tissues exists despite demonstrable physiological and immunological differences between them. For example, the adhesion mechanisms of optic tectum respond indistinguishably from those of retina to a series of dissociation procedures, but the corresponding mechanisms of heart and limb bud respond differently—each in its own way—to the same dissociation procedures. Furthermore, whereas the activity of each tectal adhesion mechanism is inhibited by the Fab that inhibits selectively the activity of the corresponding retinal adhesion mechanism, neither the CI nor the CD heart cell adhesion is inhibited by either anti-retinal Fab. These physiological and immunological differences are at present of unknown significance, but they are consistent with results reported recently by Brackenbury et al [28]. Their Fab [37] which inhibits activity of the liver CD adhesion mechanism (LCAM) does not inhibit the activity of the corresponding retinal mechanism despite the fact that cells from the two tissues display Ca^{++}-dependent cross-adhesion.

Thus, the functional similarities which define the two classes of adhesion mechanism exist despite tissue-specific differences in certain of their properties. The nature of this heterogeneity is central to several unresolved questions. For instance, does the CD mechanism of retina interact directly with that of heart, or do they act independently but in parallel to cause Ca^{++}-dependent adhesions between heart and retina cells? Studies with the anti-retina Fabs suggest that retina cells cross-adhere with heart cells using the same CI or CD mechanism through which they self-adhere. While the heterogeneity of our present antibody preparations precludes more definitive conclusions at this time, we are pursuing the answers to these questions through the use of monoclonal antibodies.

To recapitulate, we have found in our studies to date that chick embryonic cells, regardless of tissue origin, cross-adhere appreciably only if they share at least one of two classes (CI or CD) of adhesion mechanism. At present, the data are consistent with the view that each such class is composed of a family of (more or less) related molecules with functional specificity (molecular interaction) as a primary common denominator. In addition, it is possible that each class contains more than one such family.

These findings raise many questions concerning the nature—indeed the meaning—of "cellular recognition." This term has been applied to a variety of phenomena: the selective interaction of lymphocytes with target cells; the histotypic sorting-out of cells within mixed aggregates; the more rapid association of many cells with others of like kind; and the selectivity of axonal migration and neuronal integration, to cite a few (eg [38]). A common assumption has been that such phenomena are straightforward expressions of "molecular recognition" events at the cell surfaces. Cells would display tissue-specific (or cell-specific) recognition sites whose specific molecular interactions would result directly in corresponding tissue- (or cell-) specific associations. This conception does not adequately explain a number of observations: 1) Cells of many

kinds, some of which never encounter each other in normal embryogenesis, cross-adhere readily when placed together. 2) Most intercellular recognition phenomena occur *despite* the continuing interaction of "unlike" cells throughout the recognition process. In mixed aggregates, for example, cell regrouping according to tissue origin occurs in spite of continued adhesion among cells from the different tissue sources. 3) Finally, the adhesive selectivity we report upon here appears to arise from the molecular specificity of two distinct classes of recognition (adhesion) systems, neither of which is "tissue-specific." Thus, while specificity of molecular interaction and selectivity of cellular interaction (cell affinity) both exist, the latter need not [39], and in the cases reported upon here do not, arise out of the former in a simple one-to-one fashion. Cell- or tissue-specific adhesion systems may of course exist in certain instances, but since many kinds of intercellular junction and extracellular ligand with adhesion-mediating roles are shared by cells of many kinds, it seems most realistic at this time to regard cells' adhesive repertoires as depending upon the display, regulation, and amounts of a limited number of such adhesion systems.

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