INTERCELLULAR ADHESIVE SELECTIVITY

III. Species Selectivity of Embryonic Liver Intercellular Adhesion

SHARON R. GRADY and EDWARD J. McGUIRE

From the Division of Molecular and Cellular Biology, National Jewish Hospital and Research Center, Denver, Colorado 80206

ABSTRACT

A species difference in the intercellular adhesive selectivity of mixtures of embryonic liver cells is reported. This is the first quantitative assessment of species differences in the intercellular adhesive properties of embryonic cells. A collecting aggregate assay, a new double-label assay procedure, and histological and autoradiographic procedures were used to elucidate the intercellular adhesive selectivity of developing mammalian and avian liver cells. Evidence is presented that the reported adhesive differences are not due to the different cell types composing the respective embryonic mammalian and avian livers. Finally, such heterologous-homotypic selectivity of adhesion is not a property of all tissues, since it is shown that developing brain cells (mesencephalon) do not exhibit the above intercellular adhesive selectivity (mammalian vs. avian). These findings provide further support for the hypothesis that generic identity as well as cell type may play an important part in determining the intercellular adhesive behavior of heterologous-homotypic mixtures of embryonic cells. A possible evolutionary divergence of morphogenetic mechanisms is discussed.

Intercellular adhesion between dissociated embryonic cells derived from the same tissue (homotypic) but different species (heterologous) has been studied in a number of laboratories. Moscona (14) found that homotypic cells from two different species (mouse and chick) would coaggregate and subsequently undergo histotypic development without selective cell adhesion (sorting out) according to the species of origin. These studies were accomplished by aggregation of heterologous mixtures of dissociated limb bud and hepatic cells from 3–5-day embryonic chicks and from 11–13-day embryonic mice followed by plasma clot cultivation of the resulting mixed aggregates. The cell arrangements were assessed by histological methods. Further studies with dissociated cells from different organs (heterotypic mixtures) but from the same species (homologous) showed that the cells sort out with an intercellular adhesive affinity based on cell type (13, 18). Similar studies have been caried out with embryonic skin (6), neural retina (15), and embryonic kidney (17).

Garber and Moscona (7) found that chick and mouse cerebrum cells, when mixed and aggregated by a rotation method (16), would form chimeric aggregates which did not sort out according to species, further indicating the relative unimportance of generic differences in tissue selective intercellular adhesion. Roth (21), using the col-

lecting aggregate assay with embryonic chick and mouse liver, heart, and neural retina cells, found the probability of adhesion between homologous-homotypic cells to be slightly greater than that between heterologous-homotypic cells. He concluded that "tissue specificity plays a larger role in the collection of cells by aggregates than does species specificity." The conclusion drawn from these earlier studies was that cell type identity was a more important factor than generic identity in determining the intercellular adhesive properties of embryonic cells.

More recently, a number of studies suggest that generic differences may play an important role in selective intercellular adhesion and/or sorting out. Burdick and Steinberg (3) found that while mouse and chick heart ventricle cells would coaggregate, they sorted out after 2 days in culture. Further work by Burdick (2) has indicated that mouse and chick liver cells may have different cell-surface recognition properties. While these cells did not sort out from one another in chimeric aggregates, they did exhibit different sorting behavior in tissue fusion studies and coaggregation experiments when paired with a third cell type, embryonic chick heart ventricle cells. Burdick (1) has also shown that mouse and chick embryonic limb bud cells exhibit different morphogenetic properties.

The above-mentioned studies present conflicting evidence on whether or not generic selectivity of cell adhesion between homotypic cells exists. This question has been investigated (by several different assay techniques) with liver and mesencephalon cells from embryonic rodents, chicks, and rabbits. The results indicate that generic selectivity of intercellular adhesion does exist among liver but not among mesencephalon cells.

MATERIALS AND METHODS

Materials

N-2-Hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (HEPES), L-ornithine, triethanolamine, dilithium carbamylphosphate, urease type IV, phenazone, deoxyribonuclease I (DNase), and diacetylmonoxime were obtained from Sigma Chemical Co. (St. Louis, Mo.). Collagenase was a product of Worthington Biochemical Corp. (Freehold, N. J.); trypsin 1:250 was obtained from Difco Laboratories (Detroit, Mich.). The following materials were obtained from Grand Island Biological Co. (Grand Island, N. Y.): medium 199, antibiotic-antimycotic solution, fetal calf serum (FCS), trypan blue vital stain, and chicken serum. [^{3}H]Leucine, $^{32}PO_4$, and Aquasol scintillation fluid were obtained from New England Nuclear (Boston, Mass.).

Media

The following media were used as indicated. Hanks' balanced salt solution was modified by the addition of 2.35 g/liter HEPES and adjusted to pH 7.2–7.4 (abbreviated H). Calcium- and magnesium-free H (CMF) was identical to this medium but the calcium and magnesium salts were omitted. The collagenase-trypsin-chick serum solution (CTC) used for tissue dissociation contained 0.1% collagenase, 0.1% trypsin 1:250, and 10% chicken serum (previously inactivated by heating for 20 min at 56°C) in CMF. Medium used for aggregation (HNCS) contained 60% medium 199 buffered with 0.015 M HEPES pH 7.2 with 0.1 N $NaHCO_3$ (35 mg/liter), 25% H, 15% inactivated chicken serum, 1% antibiotic-antimycotic solution (all [volume/volume]), and 2 μg/ml DNase I.

The arginine-free Eagle's medium (AFE) used for culture of fetal mouse hepatocytes contained the following: Eagle's minimal essential medium (MEM) minus arginine with Earle's salts, 0.6 mM L-ornithine, 1 × antibiotic-antimycotic solution, and 5% FCS previously dialyzed against three changes of 0.9% NaCl (50 ml of FCS vs. 1 liter of saline).

Collecting Aggregate Assay

The intercellular adhesion assay is essentially that of Roth et al. (22), as modified by McGuire and Burdick (12). Chick (8-day) liver and mesencephalon aggregates were prepared as previously described (12), with the CTC incubation of mesencephalon decreased from 30 to 10 min. The livers of embryonic mouse (16–18 day), rat (17–19 day), guinea pig (about 30 day), and rabbit (28 day) were dissociated by the same procedure used for chick livers with the following exception: the CTC soak was limited to 10 min for mouse, rat, and guinea pig tissue and 15 min for rabbit. Cells were collected by centrifugation for 2.5 min at 150 *g*. The resulting cell pellet was suspended in HNCS to give a cell concentration of 2–3 × 10^7 cells/ml (equivalent to approximately 0.05 ml of packed cell volume per 3 ml), and 3 ml of this suspension was used per flask for aggregation on a gyratory shaker (New Brunswick Scientific Co., Inc., New Brunswick, N. J., model G76) at 37°C and 65 rpm. Mouse mesencephalon aggregates were made by the same procedure used for chick mesencephalon aggregates.

The procedures for labeling tissues and for dissociating labeled liver tissues have been described previously (12). Labeled mesencephala were dissociated by the method used for aggregate preparation. Unless otherwise stated, three aggregates and 10^5 $^{32}PO_4$-labeled cells in 3 ml of HNCS were used per flask.

The mean number of cells collected per aggregate was calculated. Significant differences between values were

determined by the Student *t*-test, using the Wang Computer System 2200 general library program (Wang Laboratories, Inc., Tewksbury, Mass.).

Double-Label Assay

A double-label assay for selective intercellular adhesion was used in addition to the collecting aggregate assay. This assay involved mixing two types of cells, one labeled with $^{32}PO_4$ and the other with [^{3}H]leucine, and allowing them to aggregate by the usual procedure for no longer than 1 h. The $^{32}PO_4$-labeling procedure was the same as that used for the collecting aggregate assay. The [^{3}H]leucine-labeling procedure was as follows. The tissue was minced and placed in a 6-cm tissue culture dish with either 3 ml of MEM, minus leucine, containing 15% inactivated chicken serum, 1% antibiotic-antimycotic solution, and 100 μCi of [^{3}H]leucine (sp act 5.0 Ci/mmol) or, alternatively, 3 ml of HNCS containing 100 μCi of [^{3}H]leucine. If MEM was used, the tissue was incubated for about 4 h in a CO_2 incubator; if HNCS was used, the tissue was incubated on a gyratory shaker at 37°C. After labeling, the tissue was dissociated by the usual procedures for aggregate formation, and viable cell concentration was determined by hemocytometer counts with trypan blue present (12). The dissociated, labeled cells were mixed together in 25-ml DeLong flasks and allowed to aggregate on a gyratory shaker. At least 20 aggregates per flask were selected, washed, and counted individually. In general, for each experiment, the first flask contained one cell type labeled with ^{3}H and the other cell type labeled with $^{32}PO_4$. The second flask had the same cell types, but the isotopes were reversed to eliminate labeling errors. If significant differences were found in the aggregates from the two flasks, the experiment was discarded. Samples of the labeled cells, the centrifuged cell-free aggregation medium, and the aggregates were digested in 1 ml of 1 N NH_4OH and counted after the addition of 10 ml of Aquasol scintillation fluid. After corrections for background and crossover were applied, the counts per minute per cell were calculated and the data were expressed in terms of the cell ratio for each aggregate. This ratio was normalized to what would have been a random ratio as calculated from the number of cells used in the flask, making the random aggregate cell ratio 1:1 in all cases. If the two types of cells form random coaggregates, the normalized cell ratio of each aggregate will be near 1:1; however, if the two cell types aggregate selectively, there will be two populations of aggregates, one with ratios higher than 1:1 and one with ratios lower than 1:1. The distribution of ratios within a group of aggregates is log normal; the data are plotted as a histogram of the number of aggregates vs. log cell ratio at intervals of 0.5 log units.

Autoradiography

Autoradiographs were made by the following procedure. Labeled, dissociated cells were fixed overnight in 2.5% glutaraldehyde in H medium at room temperature. Cells were deposited on slides, using a cytocentrifuge (Shandon Southern Instruments Inc., Sewickley, Pa.), and allowed to dry. The slides were coated with Kodak Nuclear Track NTB2 emulsion (Eastman Kodak Co., Rochester, N. Y.) and exposed in the dark at 4°C for 3 days if the cells were ^{32}P-labeled or for 3 wk if the cells were ^{3}H labeled. After developing, the cells were stained with Weigert's hematoxylin and counterstained with eosin.

Cell Selection

The method for selection of fetal mouse liver hepatocytes was a modification of that used by Leffert and Paul (11). Fetal mouse liver (17–18-day C57BL/6J) was dissociated with CTC by the procedure described above. The dissociated cells were collected by centrifugation for 2.5 min at 150 *g*. The cell pellet was dispersed in AFE. The liver cells were plated at a concentration of two to four livers ($6–8 \times 10^7$ cells) in 5 ml of medium on 10-cm tissue culture dishes and cultured overnight in a 5% CO_2/air incubator. The hepatocytes adhered tightly to the plates and flattened while the hemopoietic precursor cells adhered very lightly. After 12-24 h in culture, the hemopoietic cells were removed from the hepatocytes by washing gently three times with phosphate-buffered saline (PBS). The remaining adherent cells were maintained in the same arginine-free medium used for the initial plating. The medium was changed every day for the 1st wk and every 2–3 days thereafter. These cells could usually be kept in culture for several weeks.

Ornithine Carbamyltransferase (OCT) Assays

Assays for OCT were carried out as described by Richardson et al. (20). The incubation mixture (0.3 ml total vol) contained crude homogenate (10–60 μg protein), 0.266 mM triethanolamine buffer pH 7.7, 2.5 mM L-ornithine-HCl, 5 mM dilithium carbamylphosphate, and 5 U of urease. The carbamylphosphate was added last to start the reaction. After incubating at 37°C, 0.5 ml of 4% trichloroacetic acid and 0.4% phosphotungstic acid solution was added, and the samples were centrifuged at 300 *g* for 10 min. A 0.5-ml aliquot of the supernate was taken for the colorimetric determination of citrulline by a slight modification of the method of Ceriotti and Gazziniga (4). To each sample, 0.5 ml of phenazone reagent (0.4 g of phenazone dissolved in 100 ml of 40% vol/vol H_2SO_4 containing 2.4 mg of $FeCl_3 \cdot 6H_2O$) and 0.125 ml of diacetylmonoxime reagent (0.5 g of diacetylmonoxime in 100 ml 5% vol/vol glacial acetic acid) were added. The samples were placed in a boiling water bath for 30 min, centrifuged at 300 *g* for 10 min, and read at 464 nm. Formation of 1 nmol of citrulline gave an absorbance change of 0.02 U under these conditions. Protein concentrations were determined by a microbiuret assay (10).

Homogenates for the OCT assay were prepared by a method similar to that of Richardson et al. (20). Whole tissue was minced, washed twice in 0.16 M NaCl, and then homogenized in the cold, in a tight-fitting Dounce homogenizer in 0.005 M triethanolamine buffer pH 7.7 for 2 min at 16 strokes/min. Cells (presumably hemopoietic precursor and nonadherent liver cells) washed from plates during the selection procedure were collected by centrifugation at 150 *g* for 3 min and homogenized as described above for several minutes until all cells were broken. Cells which adhered to the plates (presumably hepatocytes) were washed three times with 0.16 M NaCl or PBS, and then removed from the plates in 1–2 ml of 0.005 M triethanolamine buffer pH 7.7 with a rubber policeman. This suspension was then transferred to a Dounce homogenizer and homogenized as above.

RESULTS

Heterologous-Homotypic Adhesion Measured by the Collecting Aggregate Assay

The collecting aggregate adhesion assay measures the number of cells which adhere to an aggregate in a specified amount of time. This quantitative assay measures the rate of adhesion and seems to reflect the tissue selectivity of intercellular adhesion (12). Fig. 1 shows the results of a collecting aggregate assay using liver aggregates and cells from 8-day chick, 18-day mouse, 19-day rat, and 30-day guinea pig embryos. All crosses were done on the same day with the same aggregate and cell preparations. The homologous-homotypic adhesion controls indicated that all types of cells and aggregates were adhesion competent. It is clear that chick cells do not adhere to rodent aggregates, and none of the rodent cells adhere to the chick aggregates under these conditions. Less than 0.1% of the labeled cells adhered to aggregates in any of the rodent-chick or chick-rodent crosses. The *P* values were less than 0.001 for all chick-rodent and rodent-chick crosses (heterologous-homotypic) when compared to the homologous-homotypic cell-aggregate pairs. The different rodent cells did show cross adhesion. The quantitative differences are not statistically significant, with the possible exception of the adhesion of various rodent cells to mouse aggregates. *P* values for these rodent crosses were all greater than 0.05 except for mouse cells vs. guinea pig cells at 15 min and mouse cells vs. rat cells at 30 min.

A similar experiment using 8-day chick, 18-day mouse, and 28-day rabbit embryonic liver aggregates and cells is presented in Fig. 2. Mouse and rabbit liver cells adhered to each other, but rabbit cells did not interact with the chick cells. *P* values comparing chick to chick and rabbit to rabbit adhesion with chick to rabbit and rabbit to chick adhesion were all less than 0.001. However, no selectivity was seen in the various mammalian crosses.

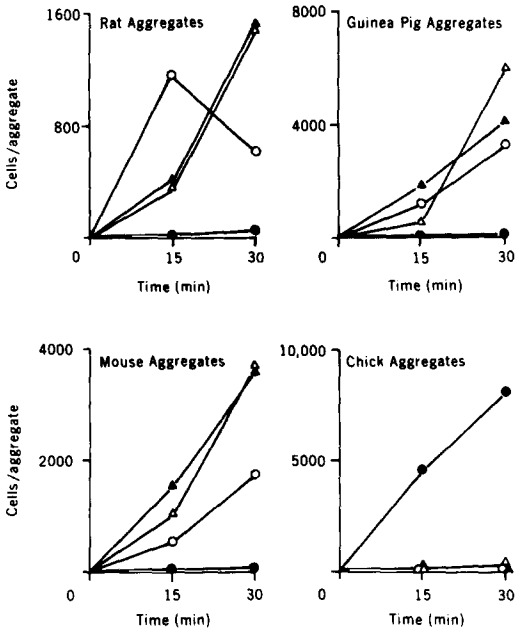

FIGURE 1 Rat, guinea pig, mouse, and chick liver adhesion, measured by the collecting aggregate assay. Livers from 19-day rat, ~30-day guinea pig, 18-day mouse, and 8-day chick embryos were used for this experiment. Each flask contained three aggregates and 10^5 labeled cells. Each point is an average of six aggregates. (○) mouse cells; (△) rat cells; (●) chick cells; (▲) guinea pig cells.

Fig. 3 is a plot of homologous and heterologous adhesion of mesencephalon cells from 8-day chick and 16- to 18-day mouse embryos. In this case there was heterologous-homotypic adhesion, and, in fact, heterologous adhesion may be somewhat greater and/or faster than homologous adhesion.

Selection, Characterization, and Adhesion of Fetal Mouse Hepatic Parenchymal Cells

A large percentage of the embryonic liver cells from rodents and other mammals are hemopoietic precursor cells (19, 23). Embryonic chick liver contains few cells of this type. It is possible that

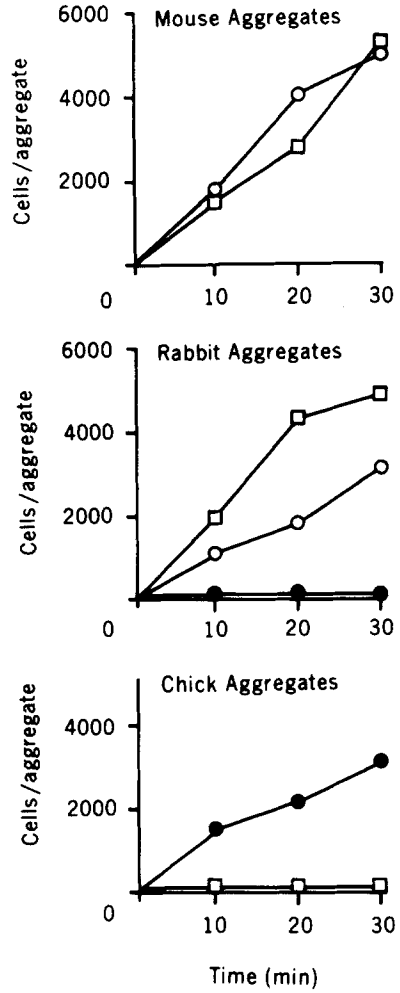

FIGURE 2 Mouse, rabbit, and chick liver adhesion measured by the collecting aggregate assay. Livers from 18-day mouse, 28-day rabbit, and 8-day chick embryos were used. Each flask contained three aggregates and 10^5 labeled cells. Each point is the average of six aggregates. (●) chick cells; (□) rabbit cells; (○) mouse cells.

the selectivity of adhesion seen in Fig. 1 is caused by differences in cell composition of the developing livers rather than true generic differences. To examine this possibility, a tissue culture selection procedure was used to remove the hemopoietic precursor cells from fetal mouse liver cell suspensions. The hepatic parenchymal cells remaining were used in a collecting aggregate adhesion assay.

The selection procedure using AFE is described in Materials and Methods. Fig. 4 shows photographs of cells before and after selection and after several days in culture. Unselected cells shown in Fig. 4*a* contain a large percentage of small hemopoietic precursor cells. After selection (Figs. 4*b*, *c*, *d*), the cells have epithelial morphology typical of hepatic parenchymal cells and untypical of fibroblasts or hemopoietic precursor cells. These selected cells are capable of growing slowly in culture for several days until confluence is reached; doubling time is 1–4 days. In addition, the cells become larger, doubling in protein content per cell by approximately 4 days in culture. This enlargement of the cells can be seen in Figs. 4*c* and 4*d* as compared to Fig. 4*b*.

These selected cells were further characterized by assaying for OCT which is found in the hepatic parenchymal cells of the fetal mouse liver but not in the hemopoietic precursors or fibroblasts. The OCT levels of whole fresh fetal liver and the selected parenchymal and hemopoietic precursor cells are listed in Table I. The plated cells had a

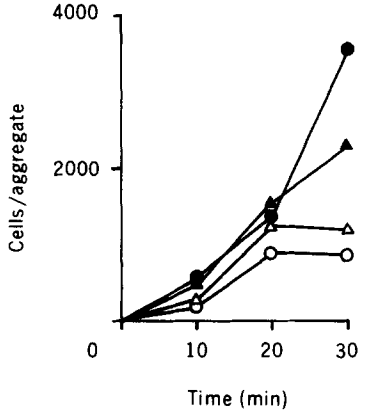

FIGURE 3 Mouse and chick mesencephalon adhesion measured by the collecting aggregate assay. 8-day chick and 16–18-day mouse mesencephala were used. Each flask contained three aggregates and 10^5 labeled cells. Each point is the mean of 12 aggregates. (○) mouse cells adhering to mouse aggregates; (△) chick cells adhering to chick aggregates; (●) chick cells adhering to mouse aggregates; (▲) mouse cells adhering to chick aggregates. *P* values are as follows:

	10 min	20 min	30 min
mouse → mouse (○) vs. mouse → chick (▲)	<0.001	0.005	<0.001
mouse → mouse (○) vs. chick → mouse (●)	<0.001	0.004	<0.001
chick → chick (△) vs. mouse → chick (▲)	<0.001	0.54	<0.001
chick → chick (△) vs. chick → mouse (●)	<0.001	0.84	<0.001

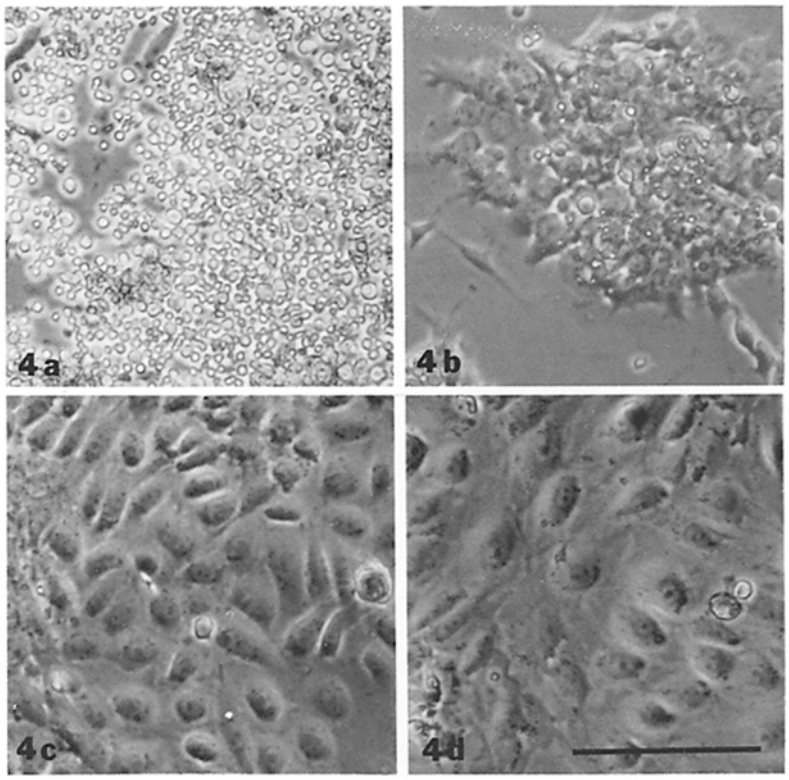

FIGURE 4 Phase-contrast light micrographs of cultured mouse liver cells. Magnification is × 250; the scale bar indicates 100 μm. (*a*) 1-day unwashed cells; (*b*) 1-day washed cells; (*c*) 4-day cells; (*d*) 11-day cells.

higher specific activity and the nonadherent cells a lower specific activity than the original liver, results which indicated that the plated cell population is enriched for parenchymal cells. The plated cells lost OCT activity in culture with a half-life of approximately 3 days.

A collecting aggregate adhesion assay using selected mouse liver cells and 17-day mouse and 8-day chick liver aggregates is shown in Fig. 5. For this experiment, the selected mouse liver cells had been kept in culture for 5 days. Similar results are obtained with cells cultured for 2–6 days. The plot shows that only 0.2% of the selected mouse liver parenchymal cells adhered to chick aggregates while 20% adhered to mouse aggregates in 30 min. In comparison, 49% of the chick cells adhered to chick aggregates and 26% of the mouse liver cells adhered to mouse aggregates in 30 min. Since the plated cells adhered to mouse liver aggregates, it appears that these cells are still adhesion competent. The P value (<0.001) indicates that the difference in adhesion of the plated cells to chick and mouse aggregates is significant. The fact that they do not adhere to chick aggregates indicates that these cells have retained their adhesive selectivity, and that this selectivity is a species selectivity and not a cell-type phenomenon.

Histological Studies

The composition of normal mouse liver aggregates provides additional evidence that cell type is not the cause of the liver selectivity seen in the collecting aggregate assay. Whole fetal mouse liver, dissociated fetal mouse liver cells, and aggregates made from these cells were fixed in 70% ethanol for histological examination. Fig. 6 shows photographs of these fixed cells. The aggregates contained all cell types in the whole and dissociated liver with the exception of mature erythrocytes, indicating that the hemopoietic precursor cells as well as the hepatic parenchymal cells were adhesion competent and that both cell types displayed "organ" selective adhesive behavior.

TABLE I

Specific Activity of OCT in Mouse Liver Cells

Cells*	μmol citrulline/mg protein/h‡
Fetal mouse liver	3.5 ± 0.5
Dissociated fetal mouse liver	3.6 ± 0.2
1-day nonadherent cells	1.0 ± 0.2
1-day plated cells	5.7 ± 0.6
2-day plated cells	4.0 ± 0.2
4-day plated cells	1.0 ± 0.1
7-day plated cells	0.5 ± 0.1

* Timed pregnant C57BL/6J mice (17 day) were used.
‡ Specific activity values reported are averages of five or more determinations with SEM. All assays were carried out under conditions of linearity with time and protein concentration.

Heterologous-Homotypic Adhesion Measured by the Double-Label Assay

The double-label assay described in Materials and Methods was carried out with the following pairs of cell types: mouse liver/chick liver, mouse mesencephalon/chick mesencephalon, and chick liver/chick mesencephalon. Autoradiographs of labeled dissociated cells showed that all types of cells except mature erythrocytes were labeled by the labeling procedures described in Materials and Methods (data not shown). Individual cells showed variable extent of labeling, but this does not appear to be dependent on cell type. Figs. 7–9 show histograms of the aggregation pairs listed above. The results were qualitatively the same as those from the collecting aggregate assay. The mouse liver/chick liver and chick liver/chick brain pairs showed two distinct groups of aggregates, one group with ratios considerably higher than the random ratio (0 in these semilog plots) and one with ratios lower than random. This indicates that these cell types did not substantially coaggregate with one another. Chick mesencephalon and mouse mesencephalon, on the other hand, did coaggregate (Fig. 8). All these aggregates have ratios very close to that predicted for a random mixture of cells.

DISCUSSION

The experiments described in this paper indicate that avian and mammalian liver cells exhibit intercellular adhesive selectivity in heterologous mixtures. This is the first report of a clear quantitative assessment of species differences in tissue selective intercellular adhesivity. Embryonic mammalian liver cells (rabbit, mouse, rat, and guinea pig) will adhere to one another, but they will not adhere to embryonic chick liver cells, and the chick liver cells will not adhere to the mammalian cells. Other workers (1–3), using more qualitative techniques, have shown that species differences play an important role in the intercellular adhesive properties of mouse and chick embryonic heart ventricle, limb bud, and liver cells. Our own studies indicate that species differences are not always primary in determining the selectivity of intercellular adhesion. Mammalian and avian brain cells (mesencephalon) adhered to one another in heterologous mixtures. This is in agreement with the findings of Garber and Moscona (7).

In the experiments reported here, 17–18-day mouse and 8-day chick embryos were used. Other investigators who have reported that chick and mouse liver cells coaggregate but subsequently fail to sort out according to species (2, 14) have used somewhat younger tissues (11–16-day mouse and

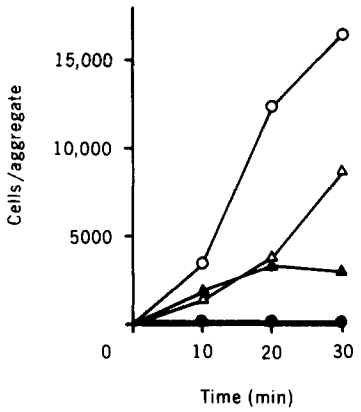

FIGURE 5 Adhesion of selected mouse liver cells measured by the collecting aggregate assay. Mouse liver cells were selected by the plating procedure described in Materials and Methods and kept in culture for 5 days. Aggregates and unselected labeled cells were from 8-day chick and 17-day mouse (C57BL/6J) livers. Selected mouse liver cells were labeled for 45 min with 0.3 mCi $^{32}PO_4$ before being removed from the plate by treatment for 15 min at 37°C with 0.01% trypsin in CMF containing 10% inactivated chicken serum. The standard collecting aggregate adhesion assay was used with three aggregates per flask and 10^5 labeled chick or mouse cells or 4.3×10^4 labeled selected mouse cells. Each point is the average of six aggregates. (○), chick cells adhering to chick aggregates; (△), mouse cells adhering to mouse aggregates; (●), selected mouse cells adhering to chick aggregates; (▲), selected mouse cells adhering to mouse aggregates.

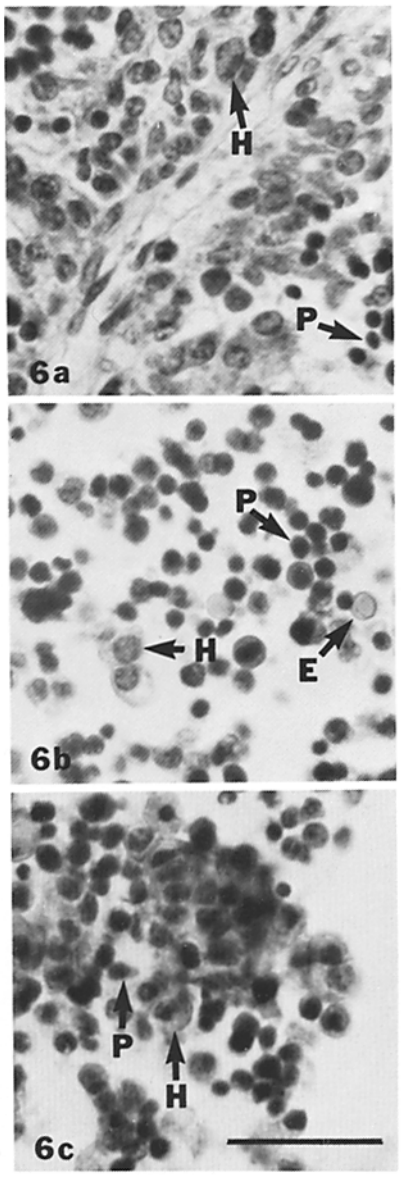

FIGURE 6 Light micrographs of fetal mouse liver. Whole, dissociated, and reaggregated fetal mouse liver preparations (18-day C57BL/6J) were fixed in 70% ethanol, dehydrated, embedded in paraffin, sectioned (6 μm), and stained with hematoxylin and eosin. Magnification is × 400; the scale bar indicates 50 μm. (*a*) whole fetal mouse liver; (*b*) CTC-dissociated fetal mouse liver; (*c*) reaggregated fetal mouse liver cells. Cell type abbreviations are: *H* = hepatocyte, *E* = mature erythrocyte, *P* = hemopoietic precursor cell.

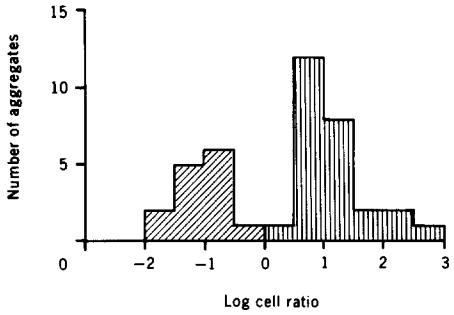

FIGURE 7 Adhesion of mouse and chick liver cells measured by the double-label assay. 8-day chick livers and 17-day mouse livers were labeled and dissociated as described in Materials and Methods. For aggregation, flask no. 1 contained 16.1×10^6 ^{32}P-labeled chick liver cells and 15.8×10^6 ^{3}H-labeled mouse liver cells. Flask no. 2 contained 11.2×10^6 ^{3}H-labeled chick cells and 12.4×10^6 ^{32}P-labeled mouse cells. Aggregation was allowed to occur for 30 min, after which time 20 aggregates were chosen from each flask. See Materials and Methods for calculation and plotting procedures. A positive log cell ratio indicates those aggregates which contained a greater number of chick cells than mouse cells. These aggregates had a mean ratio of 10 chick cells to 1 mouse cell, a median ratio of 14:1 and a range of 3–642:1 (n = 26). A negative log cell ratio indicates a larger number of mouse cells than chick cells with a mean ratio of nine mouse cells to one chick cell, a median ratio of 8:1 and a range of 3–68:1 (n = 14).

3–7-day chick). However, it has been shown that chick liver does not exhibit major temporal differences in adhesion as measured by the collecting aggregate assay (12), and the slightly older chick has the advantage of providing more liver tissue. In addition, it is difficult to match chick and mouse liver by developmental stages because of the more pronounced hemopoietic function of embryonic mouse liver. Nevertheless, the use of older tissues may account for the greater degree of selectivity shown by the experiments reported here.

In addition to embryonic age, a major difference between these experiments and those previously reported is that these experiments are measuring initial selectivity rather than observing a random aggregate which does or does not sort according to species of origin. Sorting experiments may depend on cell motility and/or a recovery period after trypsinization. Garrod and Steinberg (8, 24) have recently studied the problem of cell motility using chick liver and chick limb bud cells which do sort out in coaggregates. Their results indicate that these cells will move in a confluent

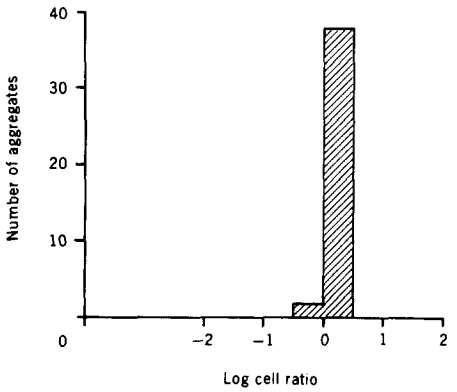

FIGURE 8 Adhesion of mouse and chick mesencephalon cells measured by the double-label assay. 8-day chick and 17-day mouse mesencephala were labeled and dissociated by usual procedures. For aggregation, flask no. 1 contained 36.2×10^6 ^{3}H-labeled mouse cells and 26.4×10^6 ^{32}P-labeled chick cells; flask no. 2 contained 22.2×10^6 ^{32}P-labeled mouse cells and 23.0×10^6 ^{3}H-labeled chick cells. Aggregation time was 1 h; 20 aggregates were chosen from each flask. A positive log cell ratio indicates a larger number of chick cells than mouse cells. The mean cell ratio is 1.3 chick cells to 1 mouse cell with a median of 1.3:1 and a range of 0.9 to 1.6:1.

culture, and therefore it is not surprising that these cells can move about in an aggregate. However, Weinstein et al. (25) have shown that normal rat liver cells do not move over the substratum as do fibroblasts in tissue culture. DiPasquale (5) has reported that most isolated epithelial cells (epidermal and corneal cells from 6.5–7-day chick embryos) do not move in culture though they do exhibit considerable surface activity. Lack of cell motility may explain why certain coaggregates fail to sort out. Our experiments measure a different parameter, i.e. selective adhesion of dissociated cells. Formation of a coaggregate is not involved, which eliminates the necessity for cell motility and, therefore, bypasses this question of cell movement in culture. It may be as a consequence of the milder dissociation conditions used that in addition to showing adhesive selectivity immediately, our cell preparations were adhesive from the time of dissociation, with little or no lag.

The double-label experiments confirm the results found by using the collecting aggregate assays. A clear intercellular adhesion selectivity is observed in heterologous mixtures of avian and mammalian liver cells. No such selectivity is seen with brain cells. This assay method utilizes freshly dissociated cells, demonstrating that selectivity is an inherent property of mildly dissociated liver. The collecting aggregate assay utilizes cell aggregates that have had time to repair their cell surfaces (1–2 h). The observed species selectivity could be a property of the recovered aggregate cell surface. The results of the double-label procedure obviate this possibility. The fact that there are a few aggregates with low cell ratios may indicate that a certain population of nonselective but adhesion-competent cells exists in our preparations. However, the selectivity may actually be greater than is indicated by these experiments, as no corrections have been made for cross-labeling by free (leaked) $^{32}PO_4$ and [^{3}H]leucine in the medium.

The embryonic mammalian liver is a major hemopoietic organ while the avian liver is not. The observed adhesive selectivity could be due to the major cell type differences—i.e., adhesive selectivity between hemopoietic precursor cells and hepatic parenchymal cells. To investigate this possibility, we have separated the embryonic mouse hepatic parenchymal and hemopoietic precursor

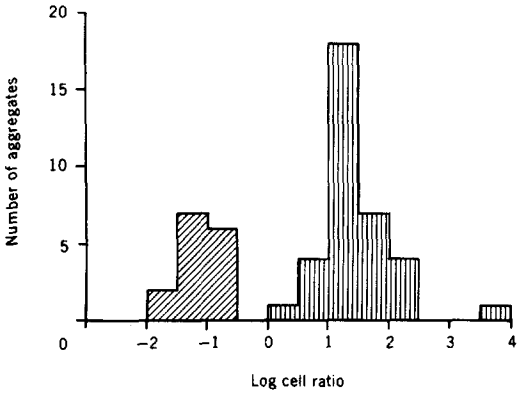

FIGURE 9 Adhesion of chick mesencephalon and chick liver cells measured by the double-label assay. Livers and mesencephala from 8-day chicks were labeled and dissociated as described. For aggregation, flask no. 1 contained 9.7×10^6 ^{3}H-labeled liver cells and 27.9×10^6 ^{32}P-labeled mesencephalon cells; flask no. 2 contained 15.6×10^6 ^{32}P-labeled liver cells and 21.6×10^6 ^{3}H-labeled mesencephalon cells. Aggregation time was 50 min; 25 aggregates were chosen from each flask. A positive log cell ratio indicates those aggregates with a larger number of liver cells than mesencephalon cells, which aggregates had a mean cell ratio of 23 liver cells to 1 mesencephalon cell with a median of 30:1 and a range of 2–297:1 (n = 35). A negative log cell ratio indicates those aggregates which contained predominantly mesencephalon cells and which had a mean cell ratio of 11 mesencephalon cells to 1 liver cell with a median of 12:1 and a range of 4–65:1 (n = 15).

cells and studied their intercellular adhesive behavior.

The selection procedure for mouse liver parenchymal cells produces a cell population after 1 day, with an increased specific activity for OCT equivalent to a 1.6-fold purification (an enzyme present primarily in the parenchymal cells). Taking into account the decay of the enzyme with time in culture (half-life of 3 days), the purification achieved at 1 day is actually 1.9-fold. Assuming that 30% of the original mouse liver cells are hepatic parenchymal cells (9, 19, 23) and that these cells are approximately three times the volume of the hemopoietic precursor cells (9), the maximum possible purification of parenchymal cells would be 1.8-fold. Therefore, by the criterion of OCT activity, the plated cell population is mostly hepatic parenchymal cells. These selected hepatic parenchymal cells do not adhere significantly to chick liver aggregates (hepatic parenchymal cells). This result indicates that the adhesive selectivity found between chick and mouse liver cells is not a cell-type difference but rather a generic intercellular selectivity.

The selected parenchymal cells do adhere to fresh mouse aggregates, demonstrating that they are adhesion competent. The selected hemopoietic precursor cells also adhere to fresh mouse liver aggregates (data not shown). Histological observation of fresh mouse liver aggregates also reveals the presence of parenchymal and hemopoietic precursor cells in the same aggregates (Fig. 6). The observation that mouse liver aggregates contain both hemopoietic precursor cells and hepatic parenchymal cells indicates that initial selectivity of adhesion does not extend to different types of cells within an organ. Both cell types exhibit "liver" selective intercellular adhesion. Presumably, other levels of selective adhesion exist which account for the fine tuning of intercellular adhesion seen within a given organ. It would appear that the above-described assays do not detect these more subtle adhesive differences between cell types derived from the same organ.

The significance of species differences in intercellular adhesive selectivity remains to be determined. A further phylogenetic investigation will reveal whether a true evolutionary divergence of morphogenetic adhesion mechanisms has occurred. These studies must also be extended to other stages of development.

If evolutionary divergence of liver "adhesion receptor" molecules can be demonstrated, it may be possible to raise xenogeneic antisera to such molecules. This would be very difficult if such molecules are conserved through evolutionary development. Further refinement of intercellular adhesion assays coupled with the above findings may reveal more subtle changes in morphogenetic adhesion molecules. The discovery of genetic polymorphism of such molecules within a single species would be an invaluable aid in unraveling the complex molecular events underlying morphogenesis.

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