*THE DEVELOPMENT IN VITRO OF CHIMERIC AGGREGATES OF DISSOCIATED EMBRYONIC CHICK AND MOUSE CELLS**

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Various embryonic tissues and organ rudiments can be dissociated into suspensions of discrete, viable cells following treatment with Ca- and Mg-free saline and trypsin.[1-3] When cultivated in vitro under appropriate conditions, such cells reaggregate into compact clusters (Figs. 1-6), which subsequently re-establish tissue-like relationships and differentiate histotypically.[2, 3] These findings, originally established for chondrogenic, nephrogenic, and myogenic cells, have recently been extended to other embryonic tissues.[3-8]

If two different types of embryonic chick cells are intermingled in the same suspension, the resulting aggregates incorporate both types of cells; however, in the course of the further development of such heterotypic aggregates,† the diverse types of cells form distinct, histogenetically uniform groupings.[2] The problem of grouping of animal cells in its relation to morphogenesis was discussed in detail by Weiss[9] in reference to the concepts of "affinities"[10] and "coaptation";[11] its experimental implications were explored in the chick embryo[12] and in amphibian embryos[13,14] and larvae[15-17] and also under conditions of tissue culture.[3, 18-20] Several of these studies strongly suggested that cells of diverse lineages manifested characteristic preferences in establishing intercellular contacts and tissue contiguity. This view was further supported by the results of recent experiments on heterotypic aggregates of chick cells[2] which convincingly demonstrated a type-specific grouping of cells in the formation and development of such aggregates. These observations fell short of proof, however, due to the difficulty of identifying early embryonic chick cells when dissociated into discrete units in suspension; under these conditions, nearly all types of such cells look alike, and their identities in heterotypic mixtures are therefore not readily determined. The obvious solution to this impasse was to have cells marked in a way which would make them individually distinguishable in a mixed population. In searching for suitable "marker cells," an attempt was made to exploit the morphological differences between chick and mouse cells; mouse cell neclei are larger than chick cell nuclei and stain differently with basic stains and hematoxylin. Previous studies have shown that mouse and chick tissues can be successfully cultured in heterologous media[21, 22] and maintained simultaneously in culture without apparent incompatibility;[23-25] it has further been noticed that under such conditions the differences of size and staining properties of the cells and nuclei of the two species are retained.

Accordingly, the feasibility of obtaining composite aggregates, consisting of both chick and mouse cells, was explored. Preliminary experiments[26] demonstrated that aggregates formed in suspensions of intermingled chick and mouse cells incorporated, under appropriate conditions, cells of both species. Upon further cultivation, such heterologous aggregates developed histogenetically in accordance with the origin of their cellular components. Due to the differences in size and the

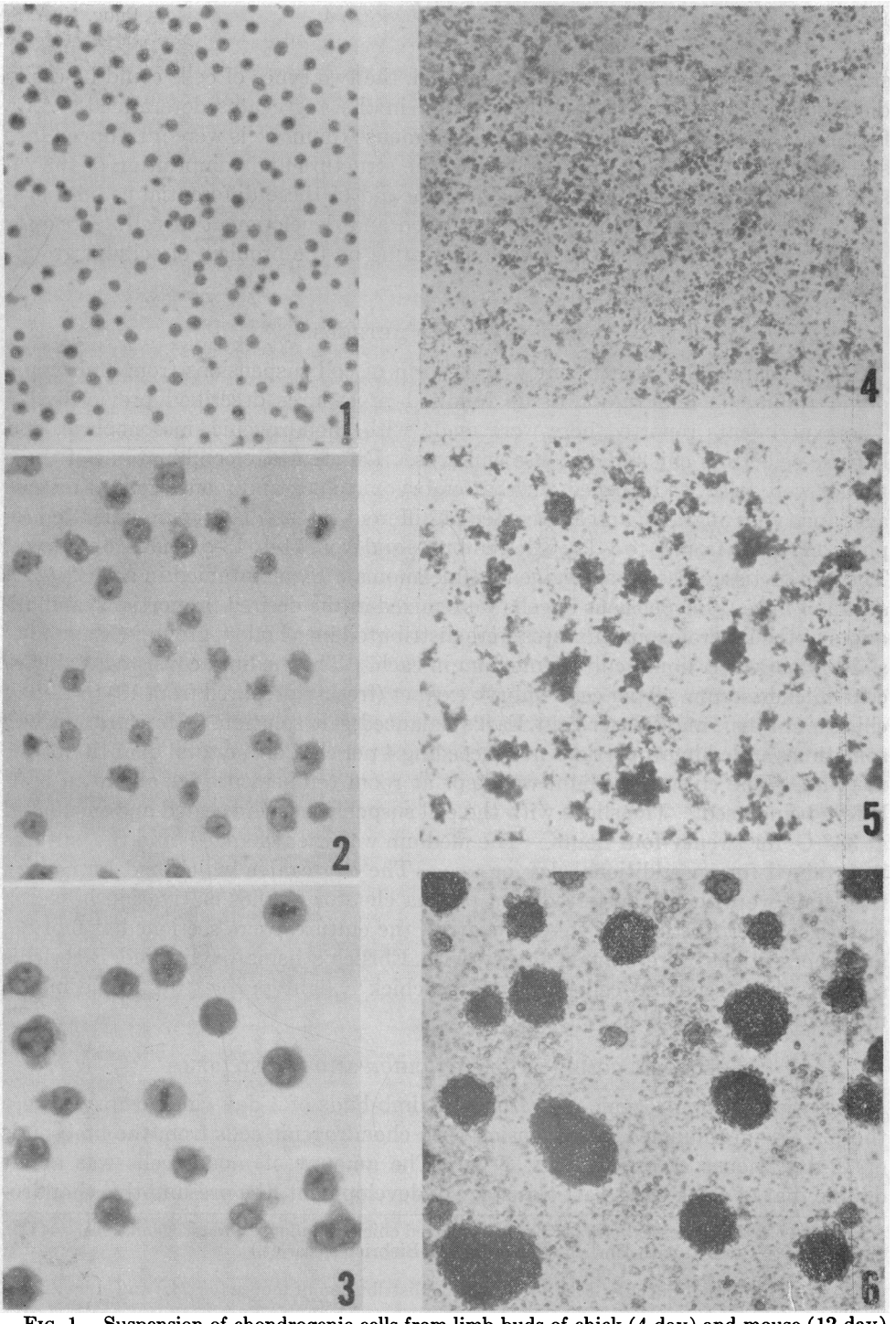


FIG. 1.—Suspension of chondrogenic cells from limb-buds of chick (4-day) and mouse (12-day) embryos. Ehrlich's hematoxylin–Biebrich's. ×160.
FIG. 2.—Suspension of chick chondrogenic cells. ×460
FIG. 3.—Suspension of mouse chondrogenic cells. ×460
FIG. 4.—Dissociated chondrogenic cells beginning to aggregate. 2-hour culture; living. ×50
FIG. 5.—A similar culture to that in Fig. 4, after 12 hours
FIG. 6.—A similar culture to that in Fig. 4, after 36 hours

staining properties of chick and mouse nuclei, the two types of cells could be easily distinguished and their precise distribution in the aggregates determined. As a further variation along this line, dissociated mouse tumor cells were introduced into suspensions of embryonic chick cells, and the structure and composition of the resulting aggregates were examined. With the aid of these differential cellular systems, various aspects of tissue reconstruction and development in cell aggregates were studied. Some of the observations bearing on the problem of cellular grouping are reported below.

MATERIALS AND METHODS

The preparation by treatment with trypsin of cell suspensions from embryonic organ rudiments and tumor tissue followed procedures described previously.[1-3] The experiments reported here were made with chondrogenic, mesonephric, and hepatic cells from chick and mouse embryos. Different age combinations of these tissues were tried, as it turned out that embryonic chick and mouse cells of diverse ages and types migrated and aggregated at different rates. This communication reports on tissues from 3- to 5-day chick embryos and from 11- to 13-day mouse embryos. The tumor tissue used was pigmented melanoma S91, maintained in a DBA/2JN strain of mice. Suspensions of cells were mixed in the desired proportions, and aliquots of the heterologous mixtures were distributed into hollow-ground (Maximow) slides with 1.0 cc. liquid culture medium in each. The medium consisted of 40 per cent chicken serum, 40 per cent embryo extract (freshly prepared from 10- to 12-day chick embryos), and 20 per cent Earl's balanced salt solution. Horse serum was sometimes added in proportions not exceeding 4 per cent of the total quantity of the medium. The culture medium was kept at room temperature for about an hour before being used. The slides with the cell suspensions were sealed and incubated at 38° C. for twenty-four hours. The medium was then changed, and the cultures maintained for an additional day or two. The aggregates which had formed by that time were then transferred to a plasma clot for further cultivation in watch glasses. After fixation in Zenker's fixative, the cultures were sectioned at 6 or 8μ, and the sections were stained briefly with Ehrlich's hematoxylin and Biebrich's scarlet, which rendered cell nuclei of the chick a light purple tint, while mouse nuclei stained a deep blue.

ISOTOPIC COMBINATIONS OF CHICK AND MOUSE CELLS

Dissociated chondrogenic cells from the limb-buds of 4-day chick embryos were thoroughly intermingled in suspension with chondrogenic cells from the limb-buds of 12-day mouse embryos (Figs. 1-3). The amount of mouse cells was about double that of chick cells. At this stage of development, the presumptive chondro-

FIG. 7.—Cartilage masses composed of interspersed chick and mouse chondrogenic cells. ×120. (FIGS. 7-19.—Stained with Ehrlich's hematoxylin–Biebrich's scarlet.)

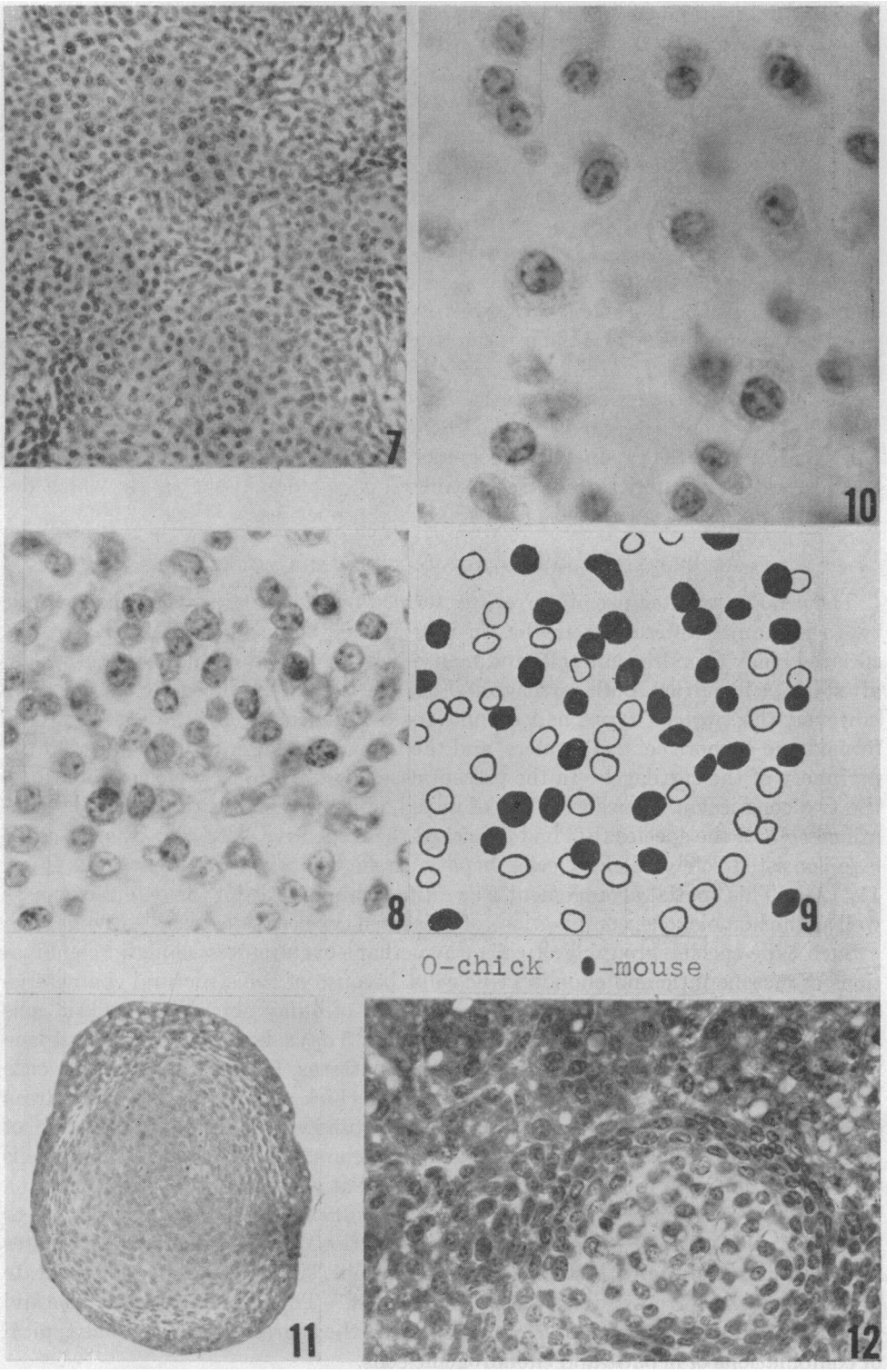
FIG. 8.—Same at ×280. Compare with Fig. 9

FIG. 9.—Outlines of nuclei of Fig. 8 to show the distribution of mouse (*circles*) and chick (*dark*) nuclei.

FIG. 10.—Full differentiated, composite cartilage, showing chick and mouse chondrocytes in a common matrix. ×980.

FIG. 11.—Aggregate of mouse liver and chick chondrogenic cells, showing a "capsule" of hepatic tissue surrounding the globule of cartilage. 4-day culture. ×80.

FIG. 12.—A 5-day culture of an aggregate of mouse hepatic and chondrogenic cells, showing the cells separated according to types. ×620.


7
10
8
9
O-chick ●-mouse
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12

blasts of the limb-bud are still in the form of stellate mesenchyme cells. The aggregates that formed in such suspensions were cultured for 6 days. Histological sections showed that they consisted of typical cartilage formed by chick and mouse cells interspersed with each other (Fig. 7). Both types of cells were intimately associated and bound by the common cartilaginous matrix into a uniform tissue fabric: the matrix surrounding a mouse cell merged quite imperceptibly with that around the chick cell next to it (Figs. 8–10). Cultures of such aggregates were maintained for periods up to one month without evidence of deterioration or incompatibility between the chick and mouse cells. Evidently the common histogenetic fabric reconstructed by the cells under these conditions was acceptable to both chick and mouse cells and suitable for their histotypical development.

An additional instance of such formative integration of interspersed chick and mouse cells was observed in combinations of liver cells. Liver tissue was obtained from 5-day chick embryos and from 13-day mouse embryos. The dissociated cells from both sources aggregated to form hepatic cords that consisted of interspersed chick and mouse cells producing glycogen or fat. In this case as well, the cells, regardless of their generic origin, reconstructed a common tissue fabric which developed in accordance with their pre-established properties.

HETEROTYPIC COMBINATIONS OF CHICK AND MOUSE CELLS

The cellular architecture of aggregates formed by cells of two diverse histogenetic types was quite different from that of isotypic cell aggregates. Mixtures of dissociated chick chondrogenic cells and mouse liver cells formed aggregates in which, after 4 days in culture, both cartilage and hepatic tissue were present. In this case, however, the two cell types had become regionally separated: the cartilage cells formed one or more central clusters, and the hepatic cells were situated around the periphery of the cartilage. In the present case, contrary to isotypic combinations, the two constituent tissues were not of mixed, chimeric composition, but each contained cells of the species that had furnished the respective cell type; that is, cartilage consisted solely of chick cells, hepatic tissue exclusively of mouse cells (Figs. 11, 12). This spatial arrangement was quite characteristic for the heterologous, as well as the homologous, combinations of these two types of cells (see also Wolff[24]).

Such type-specific grouping of cells was perhaps even more striking in combinations of mesonephric and chondrogenic cells, because of the structural characteristics of nephric tissue. In composite aggregates of 4-day chick mesonephric cells and 12-day mouse chondrogenic cells, cultured for 5 days, both kidney and cartilage cells reconstituted their recognizable tissue patterns (Figs. 13–16). The cells became consistently grouped according to type: chick chondroblasts formed areas of cartilage, mouse nephroblasts built nephric tubules. Careful examination of this material revealed no chick cells that had become chondrocytes or mouse cells that had turned into nephrocytes. Single cells that were occasionally trapped in a nonmatching environment, if they took and multiplied, developed according to their original identities. In the reversed combination of cells, namely, in aggregates of mouse mesonephric with chick chondrogenic cells, similar type-specific, separate groupings of the corresponding tissues were formed. The reconstituted nephric and chondrified showed no regular distribution within the aggregates such as was typical of combinations of hepatic and chondrogenic cells.

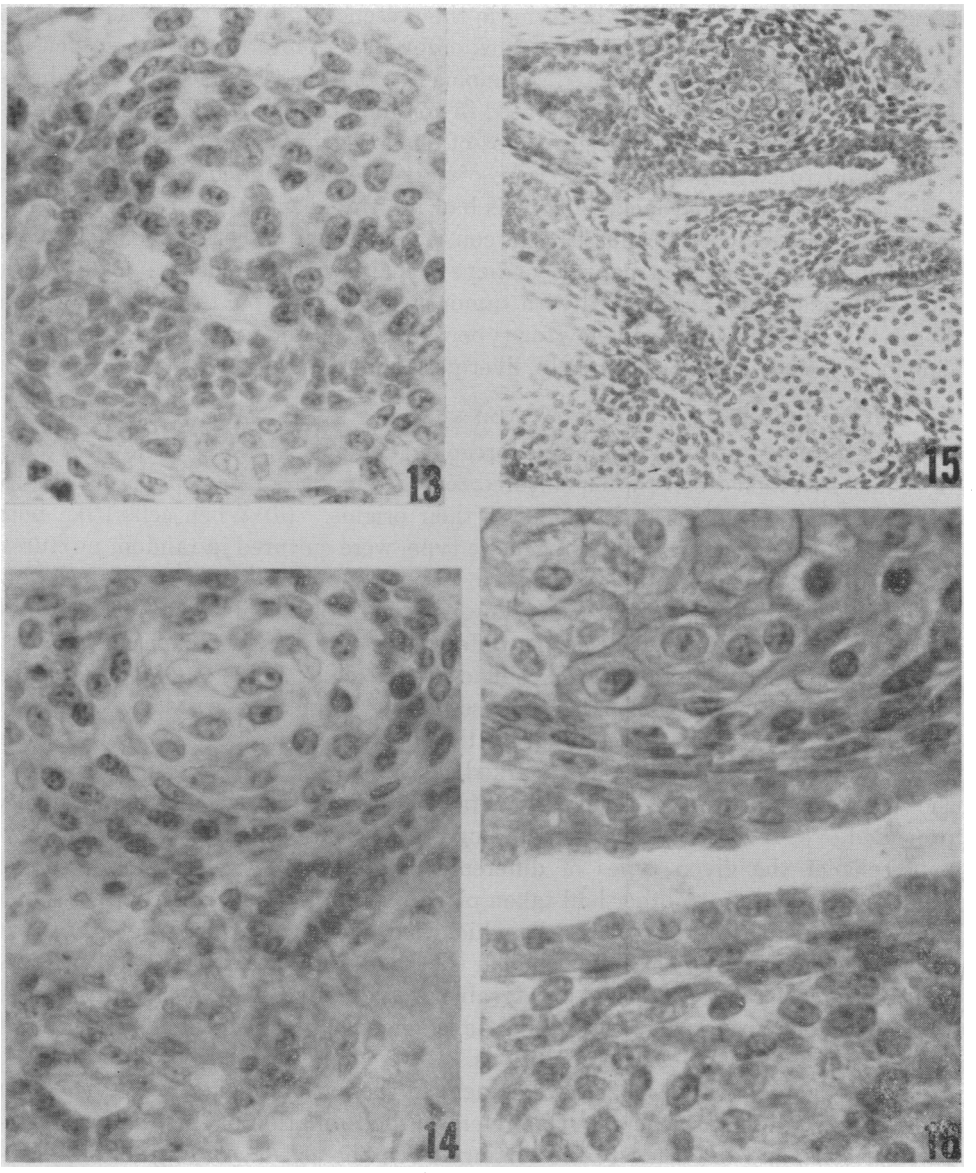

FIG. 13.—Aggregate of mouse chondrogenic and chick mesonephric cells, showing groups of cells with beginning differentiation. 3-day culture. ×250.

FIG. 14.—4-day culture of a chimeric aggregate as in Fig. 13, showing advanced histodifferentiation. ×250.

FIG. 15.—6-day culture of a chimeric aggregate as in Fig. 13, showing mosaic distribution of the cellular groupings. ×100.

FIG. 16.—Enlarged part of Fig. 15 to show the topographical proximity of the reconstituted chick and mouse tissues. ×830.

COMBINATIONS OF EMBRYONIC CHICK CELLS AND MOUSE MELANOMA CELLS

In another aspect of this study of the grouping properties of embryonic cells, observations were made on their behavior in the presence of tumor cells. Dissociated hepatic or chondrogenic cells of the chick embryo were intermingled in suspension with dissociated cells of pigmented melanoma S91 of mice (Figs. 17, 18). Embryonic and tumor cells became incorporated in common clusters, which were then further cultured for 3–5 days. Aggregates of chondrogenic chick cells and S91 cells were found to consist of a central core of cartilage surrounded by S91 cells. In older cultures, scattered melanoma cells had infiltrated into the cartilage. Aggregates of hepatic chick cells and S91 cells consisted of a central core of melanoma cells surrounded by a compact capsule of hepatic parenchyma (Fig. 19). It appears, then, that also when intermixed with tumor cells of this type, the embryonic cells clearly manifested their tendency for typewise association as well as for type-specific localization—cartilage centrally, liver peripherally.

COMMENT

The experiments reported in the foregoing demonstrated the following facts. (*a*) Chick and mouse cells, when cultured together in vitro, retained characteristics by which they could be identified as to their origins. (*b*) When cells from both species, belonging to the *same* histogenetic type, were cultured in random mixtures, they combined to form uniform chimeric tissues. (*c*) Chick and mouse cells belonging to *different* histogenetic types, however, did not readily combine but gave rise each to its discrete type-specific differentiation. Previous experiments with heterotypic cell combinations from a single species (chick embryo) had already suggested that dissociated cells tended to preserve their original type specificities and to sort out and differentiate accordingly;[2] these observations, together with the results obtained presently with cells marked clearly as to their species origin, lead to the conclusions that, under the experimental conditions explored, (1) type specificity prevailed over species specificity in guiding the association and grouping of embryonic cells of the given types of differentiation and (2) no transformation of cells of one type into another had taken place. It should be stressed at this point that these conclusions apply to cells which had evidently reached determination prior to their being dissociated, although they had not, at that time, become typically differentiated. It is thus conceivable that different results may be obtained with cells from earlier or later stages of development as well as with other types of cells, or different experimental conditions.

The problem of type-specific development in these experiments, as in the earlier ones, has two different aspects. One refers to the formation of the primary aggregates of cells, and the other to the sorting out of the cells according to kind, concurrently with or following aggregation and their subsequent differentiation. The former aspect, concerned mainly with the mechanisms of aggregation, has been only parenthetically mentioned here, and its discussion will therefore be postponed. In view of the pertinence of these problems to the observations reported, the following brief comment should be included. The formation of all types of aggregates and their histogenesis in vitro may be markedly affected by a variety of factors. Environmental changes, such as of the physical and chemical properties of the medium or the substrate, markedly influence cellular aggregation by their differential effects

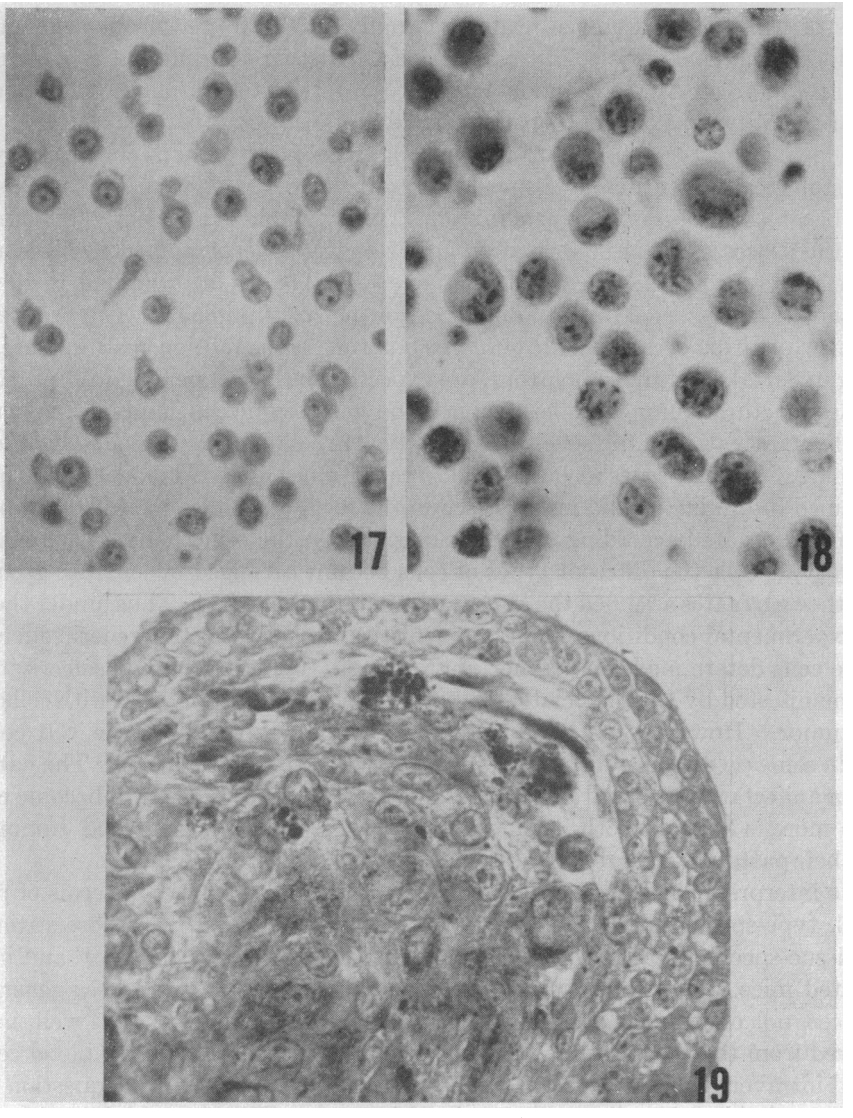

FIG. 17.—Suspension of chick liver cells. ×530
FIG. 18.—Suspension of S91 melanoma cells. ×530
FIG. 19.—Section through a composite aggregate of hepatic chick and S91 cells, cultured for 4 days and showing a "capsule" of liver tissue surrounding the cluster of melanoma cells. ×530.

on the diverse types of cells. Changes in the proportionate concentrations of different cell types intermixed in the same culture become reflected in the histological development of the ensuing aggregates.[2] As mentioned before, the rates of migration of different types of cells, as well as of cells of different generic origin, vary considerably under identical conditions. For instance, mouse mesonephric cells migrate at a slower rate than chick mesonephric cells; in cultures containing both

types of cells, this difference leads eventually to the formation of aggregates in which chick mesonephric cells predominate. Whether the different rates of migration are due to intrinsic cellular factors, to a differential response by the cells to culture conditions, or to specific activating stimuli is presently not clear.

Following formation of the primary cell aggregates, or perhaps concurrently with it, histologically identifiable tissues begin to develop, and eventually the cluster of cells becomes an organized tissue fabric. The available evidence suggests strongly that the processes of tissue formation are preceded or accompanied by a reshuffling of the aggregated cells; when more than one type of cell is incorporated in the cluster, they become sorted out to form type-specific cell groupings. The precise manner in which this occurs is still obscure, but time-lapse motion pictures, presently being undertaken in this laboratory, are expected to furnish pertinent information.

The structural differences between iso- and heterotypic cell combinations provide a striking indication of the specificities involved in cellular interactions which lead to grouping. In the tissues reconstituted from isotypic chick and mouse cells, the cells remained intermingled and interspersed in the form of cellular mosaics, without becoming segregated according to species origin. On the other hand, in aggregates of heterotypic cells the different types of cells became arranged in separate groups, so that the aggregates assumed the appearance of tissue mosaics. Thus, under the present experimental conditions, the type identities, rather than the generic identities, of the cells determined the manner of grouping. Typical grouping selectivity was also manifested by the dissociated embryonic cells when confronted with cells of the S91 tumor. However, following histogenesis of the embryonic tissue, S91 cells began, in some cases, secondarily to infiltrate between the normal cells. The nature of such manifestations, as well as the generality of such interactions, will become clearer when more is known of other combinations of dissociated normal and tumor cells and their patterns of aggregation.

The interpretation of cellular grouping in chimeric aggregates in terms of preferential, type-specific interactions between cells conforms well with observations on the tissue-specific localization of cells injected into the chick embryo[12] and into irradiated mice.[27-31] That the properties involved are effective across generic differences, not only under conditions of culture but in the organism as well, may be inferred from the successful implantations in the bone marrow of rat blood cells injected intravenously into irradiated mice.[28] In this connection, the question of the stability of chimeric cell aggregates is of interest. The successful persistence in vitro of cartilage chimeras beyond the embryonic age of their constituent cells suggested that, under such conditions, the cells, although generically alien, remained histocompatible. The response of heterologous combinations to suitable immune environments and to implantation into embryos and adults should provide additional information on the stability or the differential susceptibility of the cells under such conditions. Studies in this direction might also furnish information on the nature of histogenetic interactions between cells and the "recognition" (Weiss) effects involved, i.e., whether they function on the same basis as antibody-antigen systems[11,32,33] or whether they reflect specific properties, typical of this particular aspect of cellular behavior.

SUMMARY

1. Dissociated cells from various organ rudiments of chick and mouse embryos when intermixed in suspension cultures, readily aggregated and combined to form composite, chimeric tissues. Under suitable conditions of culture, such reconstituted tissues differentiated histotypically. This communication reports on combinations of chondrogenic, nephrogenic, and hepatogenic cells of chick and mouse embryos and S91 mouse melanoma cells.

2. In aggregates of intermixed chick and mouse cells of same type (i.e., chick and mouse chondrogenic cells) the cells reconstructed a uniform fabric which differentiated histotypically into a chimeric tissue consisting of interspersed chick and mouse cells.

3. In aggregates of intermixed chick and mouse cells of different types (i.e., chick nephrogenic and mouse chondrogenic cells) the cells became associated according to type and formed separate groupings which developed in accordance with the original histogenetic properties of the cells.

4. Due to the clear morphological differences between chick and mouse cells, it was possible precisely to identify and localize them in the chimeric aggregates. The evidence thus obtained suggested conclusively that (*a*) in the course of tissue reconstruction the dissociated embryonic cells became grouped preferentially, according to their original type identities, regardless of their generic origin, and (*b*) under the present experimental conditions no transformation of one cell type to another was observed.

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† The following terms will be used: (1) *isotypic* and (2) *heterotypic* to designate suspensions consisting of (1) predominantly one type of cell and (2) two or more cell types; (3) *homologous* and (4) *heterologous* for cells from embryos of the same specie (3) or (4) a mixture of cells from two species (i.e., chick and mouse cells).

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