Analysis of Limb Morphogenesis in a Model System

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A method for analysis of chicken limb morphogenesis was devised. This method consisted of grafting a limb ectodermal jacket containing dissociated and pelleted mesenchymal cellular components to the host somites. Different cellular components stuffed into the ectoderm could be mixed in varied ratios. After 7 days the grafts were analyzed for outgrowth. Stage 19 mesoblast cells alone when treated as above gave limblike outgrowths with good digits, toes, and claws in all cases. However, mesoblasts from the proximal half of older limbs (stages 24, 25 and chondrocytes) gave no outgrowths, and those from stage 23 gave outgrowths in 9% of the cases. In mixtures of 5% stage 19 cells with 95% chondrocytes, consistent morphogenesis (i.e., in 65% of grafts) occurred. The amount of morphogenesis (size of graft and perfection of digits) was directly proportional to the amount of stage 19 cells. However, these cells mixed with proximal cells of stages 23, 24, or 25 required higher proportions for equivalent morphogenesis. To obtain morphogenesis equivalent to the 5% mixture with chondrocytes, 10% stage 19 cells were needed when mixed with proximal stage 23, 25% with proximal stage 24 and 7% with proximal stage 25. Mixtures of stage 19 cells added to nonlimb (flank, stage 19) mesoderm, formed large tumorous mounds of tissue with no limblike features.

INTRODUCTION

The limb bud presents a good model of a developing system. The relationship between the mesoblast and its ectodermal cover, and the histogenesis and cytodifferentiation of cartilage allows an investigation of several levels of organization in a self-contained and easily manipulated body part. Beginning with the pioneering work of Saunders (1948) and Zwilling (1949) the reciprocal interaction between limb bud ectoderm and mesoderm was uncovered and identified. The relationship served as a model for the analysis of the nature and the role of different tissues in construction of the limb. The apical ectoderm ridge serves as a specific inductive structure initiating and directing outgrowth and digit formation from the early bud. The mesoderm, upon which the ridge exerts its effect, in turn supports the integrity of the ridge by producing a "maintenance factor" (see review by Zwilling, 1961). More recently, experimental interest has enlightened cellular events pertinent to limb morphogenesis including: the onset of histogenesis (Searls, 1965; Medoff, 1967); the stability of differentiated cellular phenotypes in culture (Coon, 1966; Cahn and Cahn, 1966; Nameroff and Holtzer, 1967); the cellular programs being followed during morphogenesis (Fallon and Saunders, 1968); and the time-dependent decrease in the capability of cells to change their morphogenetic programs (Crosby, 1967; Cairns and Saunders, 1954; Zwilling, 1955; Zwilling, 1968; Searls and Janners, 1969, 1971; Finch and Zwilling, 1971; Singer, 1972).

The technique of independently manipulating ectoderm and mesoderm in the study of limb morphogenesis has allowed an elucidation of the developmental capa-

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bilities of combinations of nonlimb meso-
derm with limb ectoderm as well as reveal-
ing the patterns inherent in the limb
mesoderm (Zwilling, 1955, 1956a, b;
Saunders et al., 1959). More recently
Zwilling (1964) introduced a technique of
studying the organizational and histo-
genetic capacities of limb mesoblast cells
which involved removal of the mesoblast,
dissociating the cells, then pelleting and
reimplanting them in an epidermal jacket,
and finally transplanting the stuffed ecto-
derm to an ectopic flank site. Crosby
(1967) used this method to measure the
response to the apical ridge of mesoblast
cells in early stages before limb bud out-
growth (stage 13 of Hamburger and Hamil-
ton, 1951). Her results affirmed the
earlier conclusion of Zwilling (1955) and
Saunders et al. (1957) that only limb meso-
derm is capable of growth and prolifera-
tion when associated with the ridge and is
the only mesoderm capable of supporting
(or inducing regeneration of) the ridge by
producing a maintenance factor (Amprino
and Camosso, 1958; Saunders et al., 1957;
Searls and Zwilling, 1964). Moreover,
Finch and Zwilling (1971) showed that
these properties are not expressed by
mesoderm cells later than stage 24 or by
distal mesoblast tissue later than stage 26.
The transient limb properties ("limbness"),
which are typified by the response of
limb cells to the ridge resulting in growth
and morphogenesis of a limb, has been
termed by Zwilling (1968) the "morpho-
genetic phase" of limb development. The
time when the limb cells acquire the po-
tential for forming a limb precedes the
time when they become committed to a
cytodifferentiative path, for example as
measured by the first appearance of en-
zyme activity for synthesis of sulfated
mucopolysaccharides and other end prod-
ucts (Searls, 1965; Medoff, 1967). Finally,
the technique of dissociation and reim-
plantation was also used to analyze the
capacity of mesoblast cells to sort out and

form individual tissues (Crosby, 1967;
Zwilling, 1968; Singer, 1972).

The present work is a quantitative analy-
sis of the regulative capacities of meso-
blast cells using the manipulative grafting
procedures reviewed above. Young meso-
blast cells were "diluted" with cells no
longer capable of morphogenetic modula-
tion (no longer having "limbness" traits).
The results show that such quantitative
alterations elicit striking changes in
morphogenetic expressions.

MATERIALS AND METHODS

The method whereby cells are dissoci-
ated and stuffed into an ectodermal jacket
is extensively described by Finch and
Zwilling (1971). About 50–60 leg buds,
stage 19, were excised from White Leg-
horn embryos and the mesoblast was
denuded of its ectodermal covering with a
30-min treatment in a mixture of 2% tryp-
sin (Nutritional Biochemicals Corp.)
and 1% pancreatin (Nutritional Biochemi-
cals Corp.). All manipulations were done
in calcium- and magnesium-free Tyrode's
solution (CMF). The freed mesoblast was
then dissociated into a cell suspension by
further treatment with 1 ml of a 1% crys-
talline trypsin solution (Worthington
Biochemical Corp.) for 20 min (38°C). The
mixture was poured into a full test tube
(8 ml) of horse serum–Tyrode's solution
(1 : 1) and the cells were allowed to settle
to the bottom. All but the bottom milliliter
of horse serum solution was removed, and
the cellular conglomerate was further dis-
rupted with a Vortex mixer. In the dissoci-
ation of mesoblast cells from later stages,
the cells from the dense chondrogenic
regions which failed to suspend even
after increased trypsinization (up to an
hour) were removed by filtration through
a 20-mesh Nitex cloth (Tobler, Ernst
and Traber; Elmsford, New York).

For the preparation of chondrocytes
from stage 30 limbs (7 days), the legs
were minced and then incubated with

crystalline trypsin for 45 min. The tissue clusters were dropped through a column of CMF and further disrupted in the remaining 1 ml after removing the supernatant. After disruption, 4 ml of CMF was added and mixed and the remaining chunks were allowed to settle. The supernatant was discarded; the tissue pieces were then incubated in a depression dish in CMF for 30 min, and the disruption procedures were repeated. The remaining tissue was mainly cartilage rods. The rods were incubated in the crystalline trypsin solution for 1 hr at 38°C, and then dropped through a test tube of horse serum solution; the supernatant was removed, and the remainder was disrupted by Vortex agitation. The cellular suspension was then passed through Nitex cloth to remove undissociated rods. In this way it was possible to obtain 6 million chondrocytes from 12 limb buds. Viability was greater than 85% as measured by eosin exclusion.

For the preparation of flank mesenchyme cells, stage 19 embryos were removed and resected in the region between the limbs. The resected fragments were immersed in trypsin and pancreatin solution for 20 min, the ectoderm teased away and the flank mesoderm freed from the somites and the splanchnic mesoderm. The flank mesoderm was immersed in CMF for 15 min and then in a 1% solution of crystalline trypsin for 30 min. The conglomerate was then immersed in a test tube of horse serum solution and centrifuged gently (750 rpm, 5 min). The supernatant was removed, the cellular precipitate vigorously dissociated on a Vortex mixer for 20 sec, and the cells passed through a Nitex cloth. Twenty-four embryos yielded about four million cells.

After counting in a hemacytometer, the isolated mesenchymal and cartilage cells were mixed in selected proportions. Then the mixture was pelleted at 750 rpm in a conical centrifuge tube and incubated for 1 hr at 38°, during which time the pellet became firm. The pellet was removed with a sterile spatula and placed in a dish containing horse serum solution. The pellet was subdivided into fragments each of which was stuffed into an ectodermal sac which was just removed from stage 20 leg buds. The stuffed ectoblasts were then incubated in horse serum solution for 45 min at 38°C. Eggs windowed at 4 days of incubation were used for grafting. An excision of epidermis about one somite square was made over the somites of a stage 20 animal just dorsal to the wing bud. The stuffed ectoblast was grafted to the excised area with the free mesodermal area in contact with the graft site. The window was then sealed with Scotch brand tape and the egg was returned to the incubator. After 2 days the grafts were observed and hosts which rejected grafts were discarded. Grafts were observed at the seventh day and then fixed for histological analysis.

RESULTS

Mixtures of Stage 19 Mesoblast Cells and Stage 30 cartilage Cells

All the reconstituted limbs showed some outgrowth by 7 days after grafting. The outgrowths ranged from tiny projections of a few millimeters to full-sized digits with three claws. There was also examples of unorganized massive growths resembling enlarged tumorous formations. The characteristics of each graft were tabulated. Then it was excised with the surrounding host tissue, fixed in formalin, stained in methylene blue, dehydrated and cleared in methyl salicylate to reveal the cartilaginous skeleton (Finch and Zwilling, 1971). The morphogenesis which had occurred could by assayed by the presence of jointed rods and other features of skeletal structure. Cartilage nodules alone and rods without joints indicated that histogenesis but not morphogenesis had taken place.

The ratio of stage 19 mesenchyme cells to cartilage cells in a mixture determined the extent of limb outgrowth. The series of photographs in Fig. 1 depict the direct dependence of outgrowth on the ratio of stage 19 cells when mixed with chondrocytes of stage 30. Mixtures which gave "no outgrowth" were apparently resorbed by 7 days, although 2 days after grafting they appeared well vascularized and healthy. Small growths (less than 1 mm in length) or an unorganized tumorlike mass with no identifiable limb features were also not classified as outgrowths.

Stage 19 cells, called by Zwilling (1968) "competent" or "uncommitted" cells (meaning that they have not become stabilized in their cytodifferentiated properties and retain "limbness" properties) were mixed with chondrocytes in percentage variations of 0 to 30 (see Fig. 1). Table 1 indicates the percentage of grafts giving outgrowths and morphogenesis from each one of these mixtures. The salient aspect of the results is that the addition of only a few percent of the younger cells was sufficient to effect morphogenesis from a population of chondrocytes which has long lost its limb properties. Thus these older cells, having elaborated cytodifferentiated properties, would never give an outgrowth when subjected to the grafting procedure mentioned above. However, morphogenesis occurred once the competent cells surpassed a threshold ratio of somewhere between 3 and 5% of the total mixture.

Mixtures of Stage 19 Cells and Stages 23, 24, and 25 Proximal Cells

At stage 24, chondrification is only just beginning in the central areas (Searls, 1965) and the peripheral areas still show a high mitotic activity (Searls and Janners, 1971); yet morphogenesis is no longer feasible upon disruption and grafting of the proximal mesoblast although the median mesoblast still has morphogenetic properties (Finch and Zwilling, 1971). The proximal mesoblast has only recently lost its "limbness" properties, but has not yet begun to exhibit the cartilage tissue found in the stage 25 proximal mesoblast. Since a population of cells in this stage of the limb are not homogeneous with respect to cytodifferentiation as are chondrocytes from stage 30, or morphogenetic expression, as are stage 19 cells, they must contain either some components of both cell types, or an intermediate cell type with the properties of neither. If this population contained a subpopulation of stage 19 cells, then morphogenesis should result from the addition of less stage 19 cells than was needed to initiate development with the chondrocytes (i.e., 5%). This is because the stage 19 cells would be added to a substantial population of competent cells; a population which presumably did not exist in the chondrocyte population of stage 30.

To test this, dissociated cells from the stage 19 mesoblast were mixed with cells from the proximal region of various limb stages. The proximal half or third of limbs in stages 23, 24, and 25 were removed and dissociated after removal of the ectoderm. The stage 19 cells were then mixed in various proportions as already described. In this way, the percent threshold of stage 19 cells required for morphogenesis of cell mixtures with stages 23, 24, and 25 mesoblasts could be determined.

Tables 2–4 illustrate the results of these experiments. The tabulation shows that proximal cells from each of the stages 23, 24, and 25 yield outgrowths and morphogenesis when mixed with stage 19 cells. However, the amount of stage 19 cells needed to give limblike responses differs for each of the three stages. Unexpectedly, the proximal cells of stage 24 require significantly more younger cells than either stage 25 or stage 23 in order to give the same number of outgrowths. Stage 25 cells give a response with only 3% stage 19 cells added, almost the same results as done earlier with chondrocytes. Stage 23
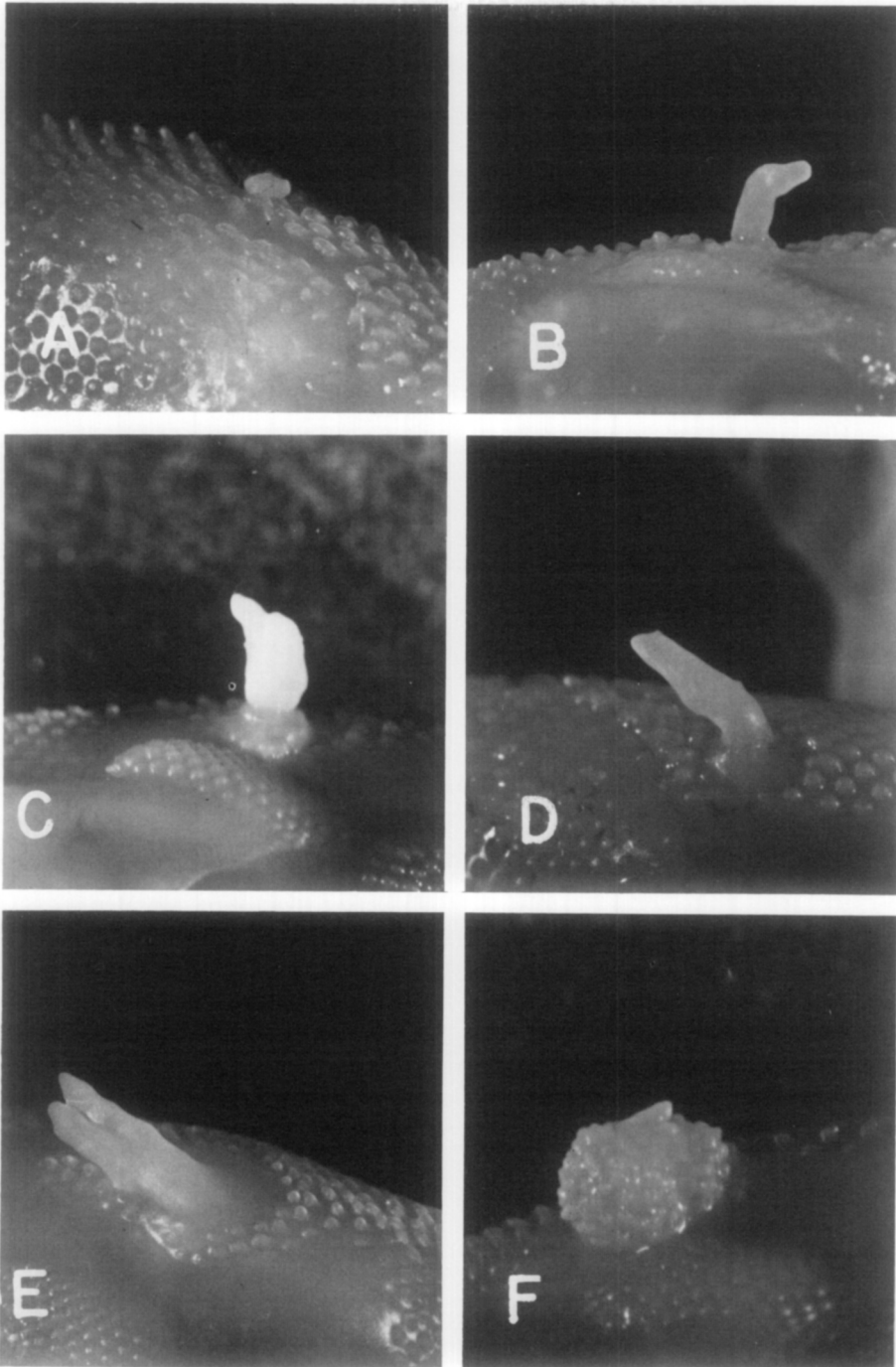
Fig. 1. Photographs of representative outgrowths fixed 7 days after grafting. (A–E) indicate dose-effect of stage 19 cells mixed with stage 30 chondrocytes. (F) reveals the tumorous outgrowth characteristic of a mixture of flank cells. All photographs enlarge material ten times. (A) 3% stage 19 cells mixed with 97% chondrocytes. (B) 5% stage 19 cells mixed with 95% chondrocytes. (C) 7% stage 19 cells mixed with 93% chondrocytes. (D) 10% stage 19 cells mixed with 90% chondrocytes. (E) 20% stage 19 cells mixed with 80% chondrocytes. (F) 50% stage 19 cells mixed with 50% flank cells.

proximal cells alone are capable of pro-
ducing an outgrowth and some morpho-
genesis, perhaps indicating presence of a
still existent stage 19 cell population. How-
ever, when stage 19 cells are added, the
number of grafts giving limblike responses
increases considerably. But the stage 24
proximal mixture with stage 19 shows few
cases of morphogenesis at the lower per-
centage. This implies that the stage 19-
like population of cells is no longer present.

Mixtures of Stage 19 Limb and Flank Mesoderm

Although the limb and flank arise from
the same general area of the lateral so-
matic mesoderm (see Crosby, 1967), they
become developmentally distinct areas
by stage 14. The flank and limb meso-
dermal cells taken from stage 19 em-

TABLE 1

RESULTANT OUTGROWTHS AND MORPHOGENESIS (PRES-
ENCE OF JOINTED CARTILAGE RODS) FROM VARIOUS
MIXTURES OF DISSOCIATED STAGE 19 MESODERM
AND STAGE 30 CHONDROCYTES STUFFED INTO ECTO-
BLASTS AND GROWN AS SOMITE GRAFTS[a]

Percent stage 19 cells in mixture	Sample size	Percent giving outgrowth	Percent giving morphogenesis
0%	8	0	0
3	35	26	26
5	22	90	65
7	24	100	65
10	22	100	67
20	10	100	70
30	9	100	70

[a] Fixed after 7 days; see Materials and Methods.

TABLE 2

RESULTANT OUTGROWTH AND MORPHOGENESIS (PRES-
ENCE OF JOINTED CARTILAGE RODS) FROM VARIOUS
MIXTURES OF DISSOCIATED STAGE 19 MESODERM
AND STAGE 23 PROXIMAL MESODERM CELLS[a]

Percent stage 19 cells in mixture	Sample size	Percent giving outgrowth	Percent giving morphogenesis
0	38	24	9
10	20	63	63
20	18	66	45
30	17	65	65

[a] Procedure as in Table 1.

TABLE 3

RESULTANT OUTGROWTHS AND MORPHOGENESIS (PRES-
ENCE OF JOINTED CARTILAGE RODS) FROM VARIOUS
MIXTURES OF DISSOCIATED STAGE 19 MESODERM
AND STAGE 24 PROXIMAL MESODERM CELLS[a]

Percent stage 19 cells in mixture	Sample size	Percent giving outgrowth	Percent giving morphogenesis
0	20	0	0
10	19	38	15
15	16	55	30
20	24	62	60
25	9	62	62
30	13	85	70

[a] Procedure as in Table 1.

TABLE 4

RESULTANT OUTGROWTHS AND MORPHOGENESIS (PRES-
ENCE OF JOINTED CARTILAGE RODS) FROM VARIOUS
MIXTURES OF DISSOCIATED STAGE 19 AND STAGE 25
PROXIMAL MESODERMAL CELLS[a]

Percent stage 19 cells in mixture	Sample size	Percent giving outgrowth	Percent giving morphogenesis
3	24	41	8
5	17	59	36
7	26	70	60
15	27	72	60
20	5	100	80

[a] Procedure as in Table 1.

TABLE 5

RESULTANT OUTGROWTHS AND MORPHOGENESIS (PRES-
ENCE OF JOINTED CARTILAGE RODS) FROM VARIOUS
MIXTURES OF DISSOCIATED STAGE 19 MESODERM AND
FLANK STAGE 19 CELLS[a]

Percent stage 19 limb cells in mixture	Sample size	Percent giving outgrowth	Percent giving morphogenesis
3	6	0	0
5	13	0	0
7	7	0	0
10	5	0	0
20	14	0	0
25	37	18	0
35	11	31	0
50	20	65	12
75	5	80	40

[a] Procedure as in Table 1.

bryos were mixed in order to determine
the effect of nonlimb mesoderm on limb
mesoderm expression. The results from
these mixtures, done as in all previous
mixtures, are reported in Table 5 and

Fig. 1F shows a typical outgrowth from such a mixture. The mixture often produced a very large cellular mound with no limb features although tiny distal outgrowths were observed and cartilage nodules were occasionally evident. Stained whole mounds of 50:50 mixtures showed no limblike internal structure. Thus the flank cells inhibit the expression of limb properties of the stage 19 cells. This inhibition further emphasizes the importance of the cellular environment for expression of the developmental capacities of the stage 19 cells, an aspect which will be elaborated further.

DISCUSSION

A system of manipulation of mesodermal limb bud cells is reported for purposes of quantitative analysis of developmental capacities of limb mesoderm. The system is artificial in that two cell populations drawn from embryos of different developmental stages are mixed which have no juxtaposition *in vivo* due to their differences in developmental age. The experimental approach is to vary the ratio of two cell populations in a mixture, only one of which is derived from a mesoblast stage capable of growth and subsequent morphogenesis when subjected to the dissociation and reassociation techniques of Zwilling (1964). We found threshold quantitative ratios of the two populations above which morphogenesis usually occurred and below which it was absent. The threshold was most striking when the two cell populations were greatly separated in stage (for example, stages 19 and 30). Only a small population of the younger cells was required to establish the threshold ratio. Therefore, quantitative alterations in the cellular interrelations elicit dramatic qualitative changes in the subsequent development of the organ.

The ability of suspended limb cells to evoke morphogenesis declines with the age of the cells. The time preceding this loss has been termed by Zwilling (1968)

the "morphogenetic phase." The present study indicates that not only are the cells competent to express this morphogenetic phase even when greatly diluted with noncompetent cells, but that this expression can be enhanced or suppressed by the particular second component in the mixture. Therefore, the cellular environment surrounding the cells from the "morphogenetic phase" determines the ultimate outcome of their expression. These quantitative and qualitative observations are expanded in the following discourse.

In the case of a mixture of stage 19 cells with cells of stage 25 or beyond, the cellular environment results in a very tentative balance between the two cell types such that morphogenesis is elicited by only a 2% shift in the population of the competent cell type (see Fig. 1A, B). One might imagine that still fewer stage 19 cells would be required to sustain morphogenesis in a mixture of stage 24 cells. Contrarily, results showed that more young cells were needed in order for morphogenesis to result. Thus, the stage 24 cells suppress the morphogenetic and growth expressions of the stage 19 cells.

The means of this suppression of morphogenesis might be conjectured at this point. Searls (1965) and Medoff (1967) showed that at the time of stage 24 the condroblasts in the limb have begun to differentiate by augmenting chondroitin sulfate production. Also at this stage, the results of Zwilling (1968) and Finch and Zwilling (1971) showed that the cells are beyond their morphogenetic phase. Stage 24 cells by themselves, unlike those of stage 23, are unable to express morphogenesis in these experimental circumstances; nor are they able to express fully a cartilage-producing function, as are the stage 25 cells. This suggests that transient cellular events occur at this stage producing a population of cells which is just losing morphogenetic abilities, and is just gaining cytodifferentiated traits.

Thrown into such a hybrid pool, the expressions of stage 19 cell capacities may no longer be possible—perhaps as a result of intercellular interferences.

Flank cells from the stage 19 embryo totally inhibit any expression of the stage 19 limb cells, until the limb cells constitute almost 75% of the mixture. This indicates that the matrix tissue around the stage 19 cells must be of limb derivation in order to effect morphogenesis. Thus, despite the similar histogenic fate of flank and limb cells (they both make muscle and cartilage *in situ*), the inhibition of morphogenetic control must be based on their morphogenetic incompatability. Crosby (1967) established the time of morphogenetic distinction of the flank cells to be approximately at stage 14. In this stage they become irreversibly distinct from those in the limb field. Although stage 19 cells cannot express their capacities when mixed with cells competent to produce nonlimb tissue, they can express such potentialities when mixed with a totally noncompetent and highly differentiated population of limb cells (chondrocytes from stage 30). This would support Zwilling's concept of "limbness" (1968), on a quantitative cellular level. It appears that morphogenesis requires all cells to be stamped with a common property (i.e., "limbness," or "flankness"). This phenomenon has also been noted by Moscona and Moscona (1965) in the suppression of feather morphogenesis by cells from other tissues, although there was no attempt to control the mixtures quantitatively. The flank cells do not inhibit the limb cells from growth because the entire graft undergoes a tremendous tumorous expansion (Fig. 1F). This uncontrolled growth is almost neoplastic. The mixing of the cell types may have released them from morphogenetic growth-control constraints, for morphogenesis ordinarily parallels limitation in growth.

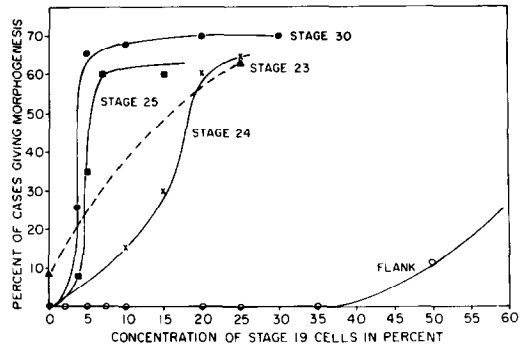The graph in Fig. 2 summarizes the relationships of the different mixtures

FIG. 2. Graph summarizing the relationship between the mixtures.

with the stage 19 cells. Although the form of the graphs are only hypothetical, it seems evident that each mixture has a characteristic curve; and all the curves seem to plateau at about 70% morphogenesis. These curves indicate that morphogenesis is determined by several factors in addition to the simple addition of cells (except for that of stage 23 which is almost linear). Perhaps an important occurrence necessary for the induction of morphogenesis is the coalescence into competent foci of the competent stage 19 cells. The time of focal formations may be critical for outgrowth because a succession of steps are involved in morphogenesis. Some of the steps already known are reciprocal interactions of the ridge and the mesodermal cells, and the chronological history of the cells.

In addition, morphogenesis is the ultimate result of an interaction of the ectodermal ridge and the limb mesoderm cells (Zwilling, 1949). Essential to this interaction may be the juxtaposition of the limb cells to the ridge. This proximity requirement may be met by cellular movements such as attraction or migration of the stage 19 cells, and could explain the complex functions exhibited in Fig. 2. These histological possibilities will be examined in a further study.

These data further extend the work done by Zwilling (1964) on the use of limb mesoderm as an experimental system for

morphogenesis. In the present study his experimental system was modified by combining populations of different developmental ages to yield a method which serves to assay the potentiality of cells to control morphogenesis. It is now possible to investigate with this technique the interactions between cells needed to elicit morphogenesis. Histological investigations could reveal the extent to which the stage 19 cells participate in the formation of the limb and the meaning of the "threshold" effect. Cell divisions of the two cell types as well as their migrations could be elucidated. Hopefully, an histological elaboration of the mechanism whereby suppression and inhibition of cell expression occurs might quantify and expose some morphogenetic mechanisms. Some of these and other problems are analyzed in a work now in preparation in which the experimental system employed in the present paper is further exploited.

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