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Experimental studies on the organization of the preimplantation mouse embryo

I. Fusion of asynchronously cleaving eggs

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SUMMARY

The interaction between mouse eggs conjoined in pairs of different ages and stages of development has been examined:

1. Forty-two per cent of chimaeras formed from 8-cell eggs paired with late morulae or early blastocysts produced morphologically normal, but large, blastocysts.
2. Fusion of pairs of vitally labelled and unlabelled eggs has shown that presumptive trophoblast derived from as late as the early blastocyst stage can become incorporated into the inner cell mass of chimaeras.

These observations suggest that the preimplantation chimaeric embryo can regulate for chronological differences in its constituent cells and that the trophoblast up to the early blastocyst stage may still be developmentally labile.

INTRODUCTION

The regulative capacity of the preimplantation mouse embryo has been demonstrated in a number of different ways. Genotypically mosaic mice have been produced by fusion of eggs up to the late morula stage (Tarkowski, 1961, 1963, 1965; Mintz, 1962*a*, 1965) and by the introduction into the cavity of one blastocyst of a few cells from another which has been disaggregated (Gardner, 1968). In addition, blastocysts have been produced as a result of the development of 1/2, 1/4 and 1/8 blastomeres (Tarkowski, 1959*a*, *b*; Tarkowski & Wroblewska, 1967; Mulnard, 1965). The blastocysts from 1/2 and 1/4 blastomeres can produce normal embryos after transfer to foster mothers but technical difficulties have so far prevented an assessment of the viability of the 1/8 blastomeres. The significance of these and other observations in relation to the development of inner cell mass and trophoblast of the mammalian blastocyst has already been reviewed by McLaren (1969) and more recently by Wilson, Bolton & Cuttler (1972).

Even after complete disruption of organization of 4- or 8-cell eggs, with a disaggregating agent, the blastomeres can reaggregate and regulate to form a blastocyst (Lin & Florence, 1970). These results have been confirmed and

extended to the blastocyst stage (Stern, 1972). The blastocyst itself can, apparently, withstand removal of substantial quantities of inner cell mass (Lin, 1969) or trophoblast material (Gardner, 1971) with little effect upon its embryo-forming capacity, although pure trophoblast or inner cell mass, cultured separately *in vitro*, do not appear to regulate to form blastocysts (Gardner, 1971). These latter observations suggest the possibility that the lability of the egg has to some extent become restricted by the blastocyst stage.

In the present study the interaction between conjoined pairs of eggs of different ages and stages of development has been examined. In addition, using the technique of vital marking devised by Wilson *et al.* (1972) to label individual blastomeres of the cleaving egg, an attempt has been made to determine the stage at which the fate of the trophoblast becomes determined.

MATERIALS AND METHODS

Nulliparous, randomly bred Q strain mice, originally obtained from the Institute of Animal Genetics in Edinburgh, were used. These were maintained under artificial conditions of 16 h light and 8 h dark. For convenience two such lighting systems, 12 h out of phase, were used so that spontaneously ovulated 8-cell eggs and late morulae/early blastocysts could be recovered simultaneously approximately 60 h *p.c.* and 72 h *p.c.* respectively. The embryos were always collected at these times, so there was a constant 12 h difference in the ages of the two components fused together, as described below. At the time of collection the older embryos were undergoing the transition from late morula to early blastocyst, some showing signs of cavitation and others not. Observations on the behaviour of transitional morulae/early blastocysts of this age following chemical disaggregation (Stern, 1972) have shown that there are no significant changes in cell properties as cavitation commences. Preliminary results from the work described also indicated that there was no apparent difference in the properties of late morulae and early blastocysts of approximately the same age. Accordingly we have drawn no distinction between them and designate them late morulae/early blastocysts (LM/EB).

All manipulations prior to culture were carried out at room temperature. Following recovery in Earles balanced salt solution (Earles BSS; Burroughs Wellcome) supplemented with bovine serum albumin (Armour Pharmaceutical Co. Ltd.), the zona pellucida was removed by placing the eggs in 0·5 % pronase (Koch–Light) in Earles BSS for up to 10 min (Mintz, 1962*b*). After several washings in the albumin supplemented medium the naked eggs were finally washed in Earles BSS supplemented with 30 % heat-inactivated calf serum (Burroughs Wellcome). Pairs of asynchronous eggs in the serum containing medium were then transferred to microdrops under liquid paraffin previously equilibrated with 10 % carbon dioxide in air, manipulated into contact with fine needles and cultured at 37 °C to the blastocyst stage. To determine whether cells

of the two components take up random positions in the fused mass and developing blastocyst, or if they segregate with respect to age, a group of fusions were performed in which one member of each pair was injected with six silicone oil drops (Wilson *et al.* 1972). Peripherally injected 8-cell eggs were then either paired immediately with late morulae or allowed to develop to late morulae before being paired with unlabelled 8-cell or late morulae. Having checked that all the drops were still in the peripheral cytoplasm of the blastomeres at the time of pairing, the eggs were allowed to fuse and develop to blastocysts when they were again examined and the final position of the drops noted.

In view of the low viability of mouse embryos transferred to foster mothers after *in vitro* fusion and culture (Bowman & McLaren, 1970) and the relatively small number of blastocysts produced, further development was studied following transfer to an *in vitro* support system consisting of a solid substrate of bovine eye-lens jelly (Jenkinson & Wilson, 1970). Fused asynchronous pairs were transferred to this system when they had developed into expanded blastocysts, 24–48 h after the start of the culture period. Thirty-six to 48 h after transfer the lens, containing the embryos, was fixed in Clarkes (absolute ethanol/acetic acid, 3:1) and embedded in paraffin wax. Sections were cut at 5 μm and stained with Ehrlich's haematoxylin and eosin.

RESULTS

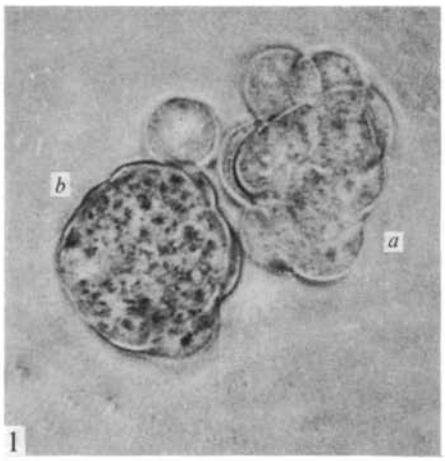
Development of conjoined pairs

Of 92 conjoined pairs of asynchronous eggs (8-cell × LM/EB), 39 (42 %) fused to form apparently morphologically normal, but giant, blastocysts (Figs. 1–4). The length of time taken for such development was somewhat variable, 32 (82 %) being observed as fully expanded blastocysts after 22 h culture and the other 7 (18 %) after 46 h; however, cavitation of the cell mass was generally observed within the cells derived, apparently, from the older partner (Fig. 3). In some of the cases where a cavity was present in the older component at the time of fusion it was noted that this persisted whilst fusion was occurring, though without time-lapse cine recordings there is no certainty that this cavity contributed to that of the mature blastocyst. In time-lapse filming of normal blastulation we have observed (unpublished) that there may be several abortive attempts at cavity formation, with collapse of the first formed small cavities, before the definitive cavity appears.

In addition, 2/92 of the conjoined eggs formed parabiotic pairs showing incomplete integration of the two components. The older component cavitated up to 24 h before the younger from which it was finally separated by what appeared to be a single layer of trophoblast.

The fate of vitally labelled cells

In this series of experiments in which pairs of eggs, 8-cell × LM/EB (Fig. 5), LM/EB × 8-cell (Fig. 6) and LM/EB × LM/EB were fused, the peripherally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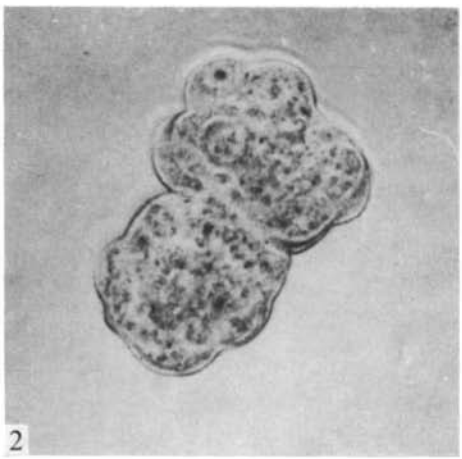
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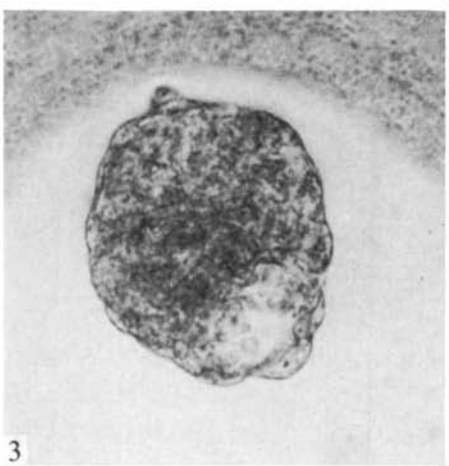
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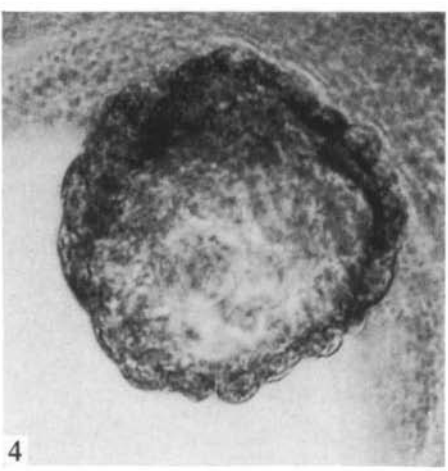
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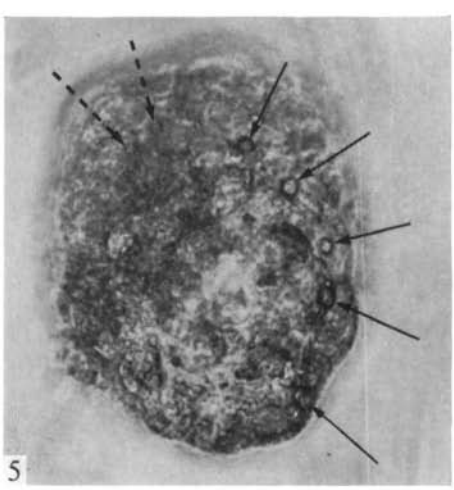
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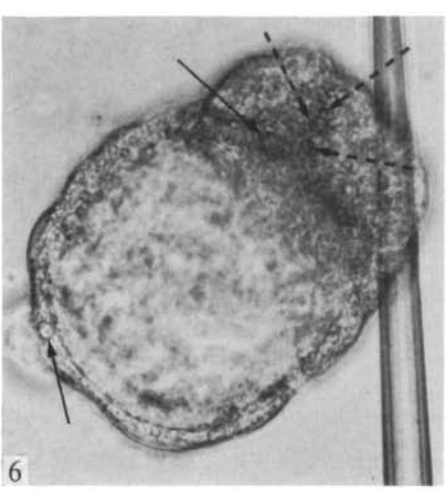
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Table 1. *Fusion of vitally labelled eggs*

Stages conjoined*	8 × 8†	8 × LM/EB	LM/EB × 8	LM/EB × LM/EB
No. of pairs fused	3	2	7	5
Total no. of drops identified after culture to the blastocyst	21	13	43	30
Position of drops‡				
(*a*) T	17	7	33	14
(*b*) ICM	4	5	10	13
(*c*) R	—	1	—	3

* The first named of each pair was injected with six silicone oil drops. Because of splitting of some drops and occasional loss of others the number found may differ slightly from the number injected.

† Taken from Wilson, Bolton & Cuttler (1972).

‡ T, Trophoblast; ICM, inner cell mass; R, rejected droplet.

injected drops of the first-named egg were found, at the blastocyst stage, both in the inner cell mass and in the trophoblast (Table 1).

Development following transfer to eye-lens jelly

Of 13 successfully transferred pairs of giant blastocysts, 11 showed signs of embryonic differentiation. Fig. 7 shows a typical early egg cylinder stage containing ectoderm, proximal and distal endoderm. Fig. 8 shows a later egg cylinder in which trophoblast proliferation has started at the embryonic pole.

DISCUSSION

Mintz (1965, 1971) has suggested that for successful formation of chimaeras the embryos to be fused should be of the same developmental age, otherwise parabiotic embryos will be formed. In the present study, although the proportion of fusions obtained from asynchronous pairs is, at 42 % considerably lower than

FIGURES 1–6

Fig. 1. An 8-cell egg (*a*) conjoined with a late morula (*b*).

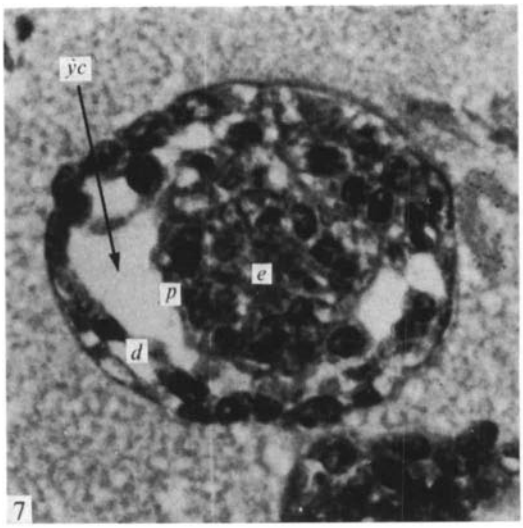
Fig. 2. Fig. 1 after 4 h in culture.

Fig. 3. Fig. 1 after 21 h in culture and showing cavitation within the cells derived, apparently, from the older partner.

Fig. 4. Blastocyst developed from Fig. 1 after 45 h in culture.

Fig. 5. Blastocyst developed from a peripherally injected 8-cell egg conjoined with an unlabelled late morula. There are 5 drops in the trophoblast (solid lines) and 1 split drop in the inner cell mass (dotted lines).

Fig. 6. Blastocyst developed from a peripherally injected 8-cell egg, cultured to the late morula and conjoined with an unlabelled 8-cell. Two drops are shown in the trophoblast (solid lines) and two, one of which has split, in the inner cell mass (dotted lines).



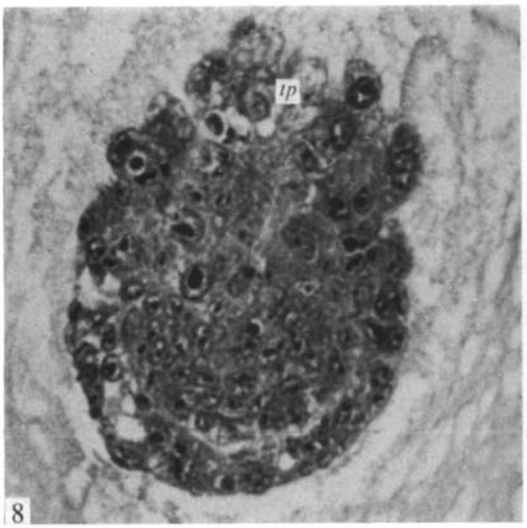


Fig. 7. Blastocyst differentiation in eye-lens jelly. Early egg cylinder stage. *e*, Ectoderm; *p*, proximal endoderm; *d*, distal endoderm; *yc*, yolk sac cavity.

Fig. 8. Blastocyst differentiation in eye-lens jelly. A later egg cylinder stage, showing trophoblast proliferation at the embryonic pole (*tp*).

the 95 % obtained from synchronous pairs by Mintz (1971), the proportion developing as parabiotic pairs (2/92) was very small so regulation is very effective. Such 'chronological' regulation has already been demonstrated for the blastocyst by transfer of an isolated 4½-day inner cell mass to a 3½-day blastocyst and the subsequent formation of an apparently normal chimaera (Gardner, 1971).

Eighty-two per cent of the successfully fusing asynchronous pairs were observed to be blastocysts after 22 h, a period of time approximating more to that taken for blastocyst production from fused synchronous late morulae than 8-cell. This suggests two possibilities. Firstly, that the older component may differentiate at an intrinsic rate to the blastocyst stage when its development becomes arrested, so allowing the younger component, developing at its own intrinsic rate, to 'catch up'. Alternatively, the older component may exert some regulatory influence accelerating the development of the younger to match its own. Under these conditions regulation in such blastocysts appears to be complete as observations on their subsequent development shows them capable at least of egg cylinder formation.

The importance of the epigenetic influence of position in the differentiation of the two cell types of the blastocyst has been suggested by Tarkowski & Wroblewska (1967) on the basis of the development of isolated blastomeres, a view substantiated by Wilson *et al.* (1972), who used a vital marking technique. An apparent lability of presumptive trophoblast and inner cell mass during early cleavage stages has also been shown by these authors with labelled 8-cell fusions (see Table 1) and was inferred by Mintz (1965). In the present study, synchro-

nous and asynchronous pairs of eggs (Table 1), in all but one case (8-cell × LM/EB), formed blastocysts with silicone-oil droplets in both the trophoblast and inner cell mass. This suggests that presumptive trophoblast, even from an early blastocyst, can be transformed into inner cell mass, though probably this occurs only in the region of apposition of the two eggs. At this interface an injected peripheral cell can get trapped in an intercellular environment and thus contribute to the inner cell mass.

Successful fusion of pairs of eggs, 12 h out of phase, has been demonstrated, on cine film, for the 4- and 8-cell egg by Mulnard (1971). Such fused pairs developed into single, apparently normal blastocysts as did the paired 8-cell and LM/EB of the present study. Both these observations (contrary to those of Mintz (1971), that conjoined asynchronous eggs develop relatively independently to produce parabiotic pairs) suggest that in addition to regulation for addition or removal of groups of cells the preimplantation mouse embryo is capable of regulation for chronological differences in its constituent cells.

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