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Investigation of the fate of 4·5 day *post-coitum* mouse inner cell mass cells by blastocyst injection

By R. L. GARDNER[1] AND J. ROSSANT[2]

From the Department of Zoology, Oxford University

SUMMARY

Two distinct patterns of chimaerism were found in conceptuses produced by injecting dissociated 4·5-day inner cell mass cells into genetically dissimilar blastocysts. Pattern 1: donor cells were found in the endoderm layer of the visceral yolk sac, but not in the adjacent mesoderm layer of this organ or in the foetus itself. Pattern 2: donor cells were found in the mesoderm layer of the visceral yolk sac and/or foetus, but never in the yolk-sac endoderm as well. Primitive endoderm cells of donor inner cell masses are responsible for the first pattern and primitive ectoderm cells for the second. These results, together with those of previous studies, suggest that the entire foetus, including its endodermal components, is formed from the primitive ectoderm, and that primitive endoderm forms only extra-embryonic endoderm of the conceptus.

INTRODUCTION

Following blastulation the mouse embryo is composed of an outer monolayer of trophectoderm and enclosed cluster of inner cell mass (ICM) cells. By the late blastocyst stage trophectoderm cells surrounding the blastocoel begin to enlarge relative to the remainder, and a distinct layer of endoderm forms on the blastocoelic surface of the ICM (Dickson, 1966; Snell & Stevens, 1966). These endoderm cells (primitive endoderm cells) are eventually separated from the remaining ICM cells (primitive ectoderm cells) by a basement lamina (Enders, 1971). In conventional histological preparations they tend to stain more intensely than other cells of day-5 blastocysts (e.g. Snell & Stevens, 1966), and also differ ultrastructurally in possessing conspicuous endoplasmic reticular cisternae (Enders, 1971; Enders, Given & Schlafke, 1978). Furthermore, living cells obtained by enzymatic dissociation of microsurgically isolated primitive endoderm exhibit a 'rough' appearance contrasting with the relatively 'smooth' outline of similarly isolated primitive ectoderm cells (Gardner & Papaioannou, 1975).

In an earlier series of experiments the developmental potential of 4·5-day

[1] *Author's address (for reprints):* Department of Zoology, University of Oxford, South Parks Road, Oxford, OX1 3PS, U.K.

[2] *Author's address:* Department of Biological Sciences, Brock University, St. Catharines, Ontario, Canada, L1S 3AI.

post-coitum (*p.c.*) primitive endoderm and primitive ectoderm was examined by injecting either microsurgically isolated tissue fragments or dissociated R (rough) versus S (smooth) cells into genetically dissimilar host blastocysts. The results of analysis of 39 chimaeric conceptuses were consistent with the hypothesis that primitive endoderm cells formed only extra-embryonic endoderm, while those of the primitive ectoderm formed the entire foetus including its endoderm, as well as the extra-embryonic mesoderm (Gardner & Papaioannou, 1975). However, the conclusions were necessarily tentative because visceral yolk sacs were not separated into their constituent endodermal and mesodermal layers prior to analysis.

Further investigation of the potency of 4·5-day *p.c.* ICM cells reported in this paper was prompted by the finding that the visceral yolk sac can be dissected into its constituent endoderm and mesoderm following enzymic treatment. In addition, emphasis was placed on ensuring as high a donor cell contribution as possible in the present study, and on testing morphologically intermediate cells.

MATERIALS AND METHODS

As previously, genetically determined electrophoretic variants of the enzyme glucophosphate isomerase (GPI) were used as cell markers. Donor blastocysts were genotypically *Gpi-1*^{b}/*Gpi-1*^{b} and host blastocysts *Gpi-1*^{a}/*Gpi-1*^{a} in all except one limited series of injections in which the reciprocal combination was employed. They were obtained by natural matings of mice belonging to random-bred, inbred or hybrid stocks (see Tables 1 and 3 for details). The mice were maintained on a regime of 12 h light/12 h dark. Assuming mating to have occurred at the mid-point of the dark period, donor and host blastocysts were recovered by flushing excised uteri between 109 and 113 h and between 86 and 89 h *p.c.*, respectively. PBI medium (Whittingham & Wales, 1969) containing 10 % (v/v) heat-inactivated foetal calf serum was used for the recovery, manipulation, short-term culture, and *in vivo* transplantation of embryos.

Microsurgery

ICMs were dissected from donor blastocysts with the aid of a Leitz micromanipulator assembly (Gardner & Johnson, 1972). They were dissociated in the single cell injection experiments by pipetting, after exposure to 0·25 % trypsin (Difco, 1/250) for approximately 30 min at 37 °C (Cole & Paul, 1965). The dissociation procedure was later modified in the multiple cell injections, by reducing incubation in trypsin to 15 min and preceding it with 10 min exposure to 0·5 % pronase (Calbiochem. grade B) in phosphate-buffered saline (PBS), similarly at 37 °C. Injection of the three morphological classes of cells into the blastocoelic cavity of host blastocysts was carried out by both authors using the method described fully elsewhere (Gardner, 1978).

In some experiments single cells were transplanted, while in the remainder several were injected into each host blastocyst. Typically, between 2 and 5 R or S cells were injected in the latter experiments, except in a single series in which the number per blastocyst was raised to 8–10. Operated blastocysts were cultured for at least 1 h before being transplanted to one or both uterine horns of females of *Gpi-1^a/Gpi-1^a* or occasionally *Gpi-1^a/Gpi-1^b* genotype on the third day of pseudopregnancy.

Analysis of conceptuses

Recipients were killed between the 15th and 18th day *p.c.*, mainly on the 16th day. Conceptuses obtained in the single cell injection experiments were dissected to provide the foetus and visceral yolk sac for electrophoretic analysis. While the former was analysed as a single fraction, the latter was separated into constituent endoderm and mesoderm as follows. After a rinse in PBS, visceral yolk sacs were incubated in a mixture of 0·5 % trypsin and 2·5 % pancreatin (Difco) in calcium-magnesium-free Tyrode saline at pH 7·7 for a minimum of 3 h at 4 °C (Levak-Svajger, Svajger & Skreb, 1969). Thereafter, they were transferred to PBS at room temperature, and the endoderm was peeled away from the mesoderm with watchmaker's forceps. The two layers parted readily in the bulk of the organ, resistance being experienced only in the zone that adjoins the placenta *in situ*, which is thrown into a series of tight folds or pleats. Visceral yolk sacs were thus analysed as three fractions; endoderm, mesoderm, and unseparated residue. The visceral yolk sacs of conceptuses produced in the multiple cell injection experiments were also treated as above. However, chorio-allantoic placenta, and amnion plus umbilical cord, were analysed as additional fractions, as were liver, gut, and lungs of each foetus.

All dissected fractions were rinsed in PBS, diluted with a small volume of distilled water, then frozen and thawed prior to horizontal starch gel electrophoresis (Chapman, Whitten & Ruddle, 1971; Gardner, Papaioannou & Barton, 1973).

It is difficult to establish the sensitivity of this electrophoretic assay for small and often variable-sized pieces of conceptuses. In artificial mixtures of certain embryonic fractions of *Gpi-1^a/Gpi-1^a* and *Gpi-1^b/Gpi-1^b* origin, cells of the latter genotype can be detected consistently when comprising 3 % of the sample (Gardner *et al.* 1973; Gardner, unpublished observations). However, enzyme activity per cell may vary from tissue to tissue (Dewey, Gervais & Mintz, 1976).

Histology and histochemistry of visceral yolk sacs

Several visceral yolk sacs that had been exposed to the trypsin-pancreatin mixture were fixed in Bouin's fluid for wax embedding, sectioning, and staining with haemalum and eosin. Additional isolated endodermal and mesodermal

fragments were treated as described by Orchardson & McGadey (1970) for *in situ* visualization of GPI activity.

RESULTS

Investigation of trypsin-pancreatin-treated visceral yolk sacs

Examination of histological sections of several treated yolk sacs revealed areas where endoderm had detached spontaneously from mesoderm. In these regions, and also the much more extensive ones produced by dissection, separation was found to be very clean. Histochemical staining of endodermal and mesodermal fragments isolated from additional yolk sacs demonstrated that both retained substantial GPI activity. Judging by visual inspection at low magnification, enzyme activity seemed to have a uniform regional distribution in both components.

Injection of single cells

Initially, the R versus S cell distinction was considerably less obvious in this than in the earlier study. Therefore, only one cell was injected into each host blastocyst in the first series of experiments. Donor cells were assigned to one of three classes; R cells, S cells, and cells of intermediate morphology (I cells). The rate of development of injected blastocysts was comparable for each class of donor cell, though the frequency of chimaeras was higher in the I, than the R or S cell injections (Table 1).

Details of the distribution of donor cell clones in the visceral yolk-sac fractions and foetuses of the 13 chimaeras are presented in Table 2. Two chimaeras are uninformative (Nos. 9 and 11, Table 2), because in each case the clone was confined to the unseparated residue of the yolk sac. The remaining 11 can be assigned unequivocally to one or other of two categories on the basis of distribution of donor cell clones between the endodermal and mesodermal fractions of the yolk sac, and the foetus. Those in the first category (type A chimaeras, Nos. 2, 3, 10 and 12, Table 2) exhibit restriction of donor cells to the endoderm fraction of the yolk sac, and those in the second (type B chimaeras, Nos. 1, 4, 5, 6, 7, 8 and 13, Table 2), restriction to the mesodermal fraction and/or foetus.

Visual estimates of the contribution by donor cells to chimaeric fractions are also displayed in Table 2. They indicate that donor cells were in the minority in every case. Only in two fractions did they exceed 25 %, and in more than half they were estimated to be less than 10 %. Furthermore, as noted earlier, in two conceptuses the clones were detected only in the yolk-sac residue. These findings raise the question whether type A versus B patterns of chimaerism are simply an artifact due to limited size of the donor clones and sensitivity of the GPI assay.

An obvious way of attempting to increase the contribution made by donor

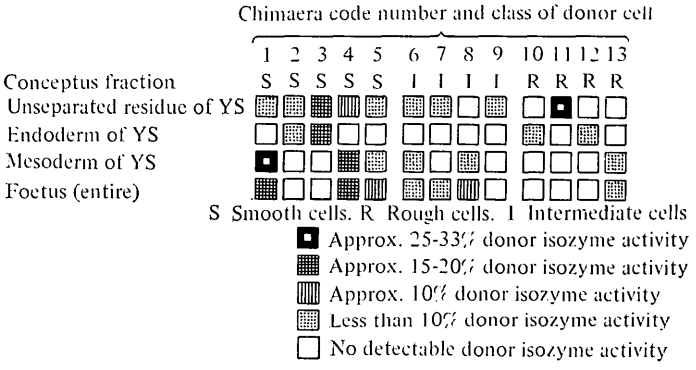
Table 1. *Development of conceptuses injected with single 4·5-day ICM cells of different morphological classes*

Phenotype of donor cell	Derivation of blastocysts: Donor	Derivation of blastocysts: Host	Number of operated blastocysts*	Living conceptuses: Total	Living conceptuses: Chimaeric	Chimaera code numbers
Smooth	(CBA × C57/BL) × (CBA × C57/BL)	Mixed CFLP + PO	34	28	2	1, 2
	(CBA × C57/BL) × (CBA × C57/BL)	PO	9	6	0	
	AG/Cam × AG/Cam	CFLP	7	7	3	3, 4, 5
			50	41	5	
Intermediate	(CBA × C57/BL) × (CBA × C57/BL)	PO	5	4	1	9
	AG/Cam × AG/Cam	PO	7	5	0	
	AG/Cam × AG/Cam	CFLP	6	6	3	6, 7, 8
			18	15	4	
Rough	(CBA × C57/BL) × (CBA × C57/BL)	CFLP	17	16	1	10
	(CBA × C57/BL) × (CBA × C57/BL)	PO	7	5	2	12, 13
	AG/Cam × AG/Cam	CFLP	4	4	1	11
			28	25	4	

Mouse stocks: AG/Cam inbred stock (see Staats, 1972), CFLP random-bred albino stock (Anglia Laboratory Animals Ltd.), PO random-bred albino stock (Sir William Dunn School of Pathology, Oxford).

* Number of operated blastocysts transferred to recipients that became pregnant.

Table 2. *Distribution and approximate proportions of donor GPI activity in chimaeras produced by injecting single 4·5-day* p.c. *ICM cells into blastocysts*



cells is to inject several into each blastocyst. However, an obstacle to multiple cell injections was the poor correlation between morphology of the transplanted cells and the pattern of chimaerism they produced in the foregoing experiments. Thus while all three informative chimaeras produced by injecting single I cells were type B, those in the R and S cell series included both type A and type B (Table 2).

Multiple cell injections

Several modifications of procedure for dissociating donor ICMs were explored to determine whether the R versus S cell distinction could be enhanced. Two of these modifications consistently yielded clearer resolution of the dissociated cells into two populations. The first was to reduce exposure to trypsin to 15 min and precede it by 10 min incubation in 0·5 % pronase in PBS. This considerably reduced the amount of pipetting required to achieve final dissociation. The second was to leave the dissociated cells for several minutes either at room temperature or at 37 °C before selection (Fig. 1).

Only the roughest and smoothest cells were selected for injection, the number transplanted to each blastocyst ranging from two to ten in different experiments (Table 3). Gut, liver and lungs were analysed as separate fractions in all conceptuses produced in these experiments. Details of the experiments, including rates of development of injected blastocysts and frequencies of chimaeras, are summarized in Table 3. Table 4 summarizes the results of GPI analysis of the 24 chimaeras. Three points of relevance are evident from this table. First, the donor contribution to chimaeric fractions is generally high. It was estimated to be 25 % or more in 40 % of the fractions, and around 50 % in approximately 25 % of them. Second, 22 chimaeras could be classified unequivocally, 11 as type A and 11 as type B. In the remaining two, chimaerism was detectable only in the yolk-sac residue and placenta (Nos. 31 and 37), precluding classi-

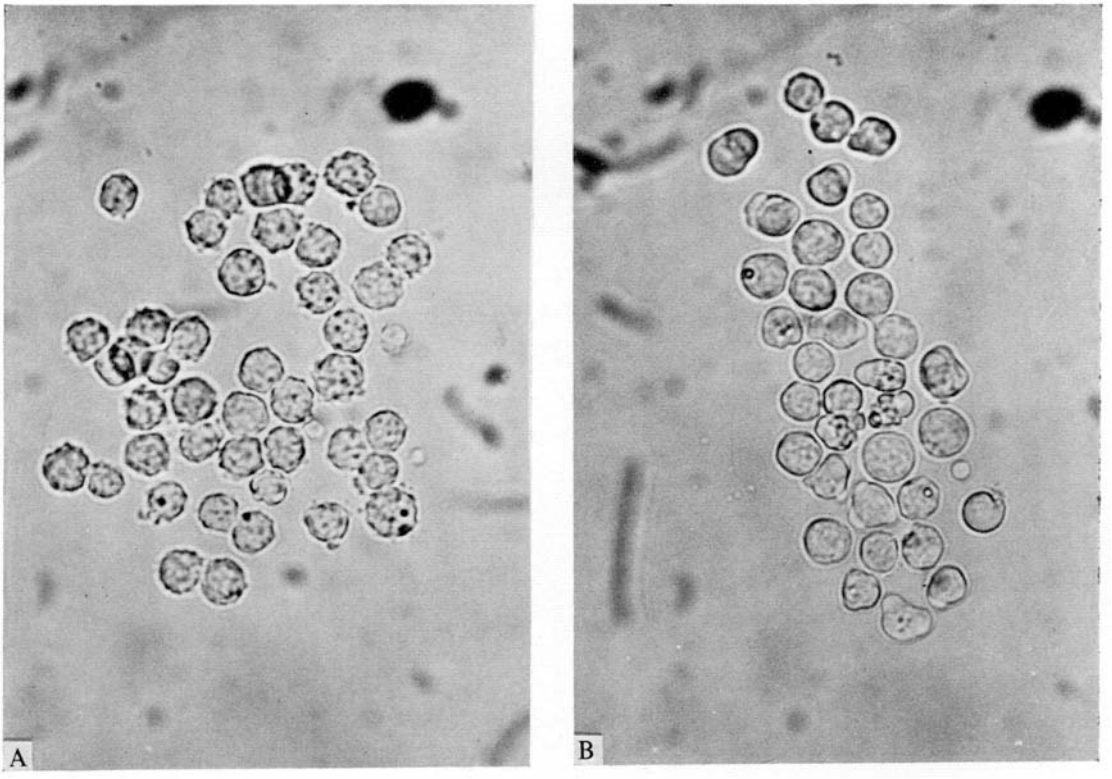


Fig. 1. Groups of (A) R cells, and (B) S cells from a donor ICM which was incubated in 0·5 % pronase for 10 min followed by 0·25 % trypsin for 15 min dissociated, and then cultured for 30 min in PBI plus serum prior to cell selection (× 400).

fication. Finally, all A type chimaeras developed from blastocysts receiving R cells and all type B from blastocysts receiving S cells.

Distribution of R and S cells in donor ICMs

Various attempts were made to separate primitive endoderm from ectoderm prior to dissociation in order to check the distribution of unequivocal R and S cells between these tissues. Incubating punctured blastocysts for approximately 30 min at 4 °C in the trypsin/pancreatin mixture used for visceral yolk-sac tissue separation (see Materials and Methods) was found to assist subsequent micro-dissection. Judging by continuous visual inspection throughout microsurgery, clean separation of the two layers was achieved in six blastocysts. The isolated endodermal and ectodermal fragments were incubated individually first in pronase and then trypsin, dissociated, coded, and finally scored after brief incubation in fresh PBI medium plus serum. The results, presented in Table 5, show that unequivocal R cells are confined exclusively to the primitive endoderm, and that the overwhelming majority of S cells are located in the ectodermal fragments. The presence of occasional S cells

Table 3. *Development of blastocysts injected with multiple R or S cells*

Phenotype of donor cells	Derivation of blastocysts		Number of cells injected/ blastocyst	Number of operated blastocysts*	Living conceptuses		Code number of chimaeras
	Donor	Host			Total	Chimaeric	
S (Smooth)	PO	CFLP	2–4	24	23	4	14, 15, 16, 22
	CFLP	PO	5	10	6	3	17, 18, 19
	PO	PO	3–4	8	7	1	20
	PO	PO	4	4	2	1	21
	PO	PO × CFLP	8–10	5	3	2	23, 24
				51	41	11	
R (Rough)	PO	CFLP	2–4	45	35	6	25, 26, 29, 30, 31, 32
	CFLP	PO	5	7	3	1	27
	PO	PO	3–4	6	2	1	28
	PO	PO	4	3	2	0	
	PO	PO + CFLP (mixed)	4	6	4	0	
	PO	PO × CFLP	8–10	5	5	5	33, 34, 35, 36, 37
				72	51	13	

* Number of operated blastocysts transferred to recipients that became pregnant.

Table 4. *Distribution and approximate proportions of donor GPI activity in chimaeras produced by injecting multiple 4·5-day* p.c. *ICM cells into blastocysts*

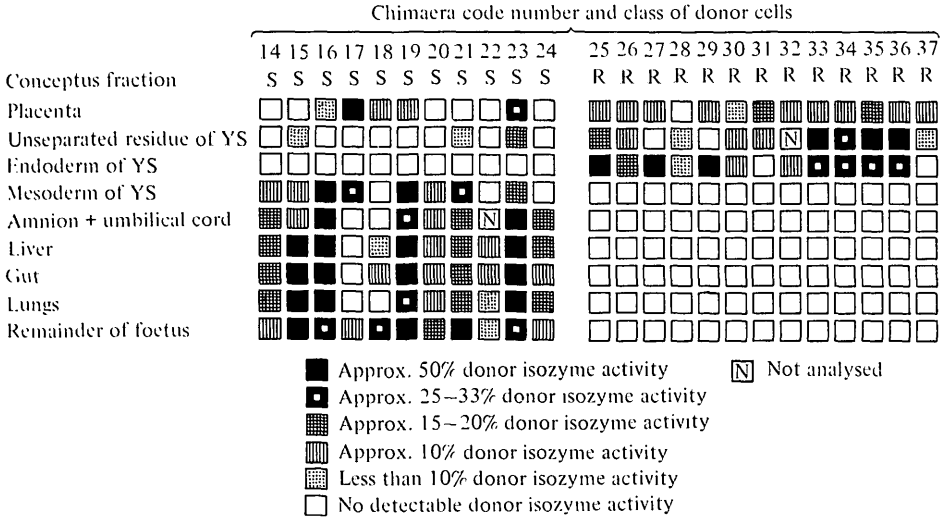


Table 5. *Distribution of R versus S cells in 4·5-day ICMs*

	Tissue fragment	Total no. of cells	No. and (%) R cells	No. and (%) S cells	No. and (%) ?(I) cells
ICM 1	Ectoderm	25	0 (0)	25 (100)	0 (0)
	Endoderm	42	40 (95)	0 (0)	2 (5)
ICM 2	Ectoderm	35	0 (0)	32 (91)	3 (9)
	Endoderm	44	42 (95)	2 (5)	0 (0)
ICM 3	Ectoderm	23	0 (0)	23 (100)	0 (0)
	Endoderm	56	43 (77)	1 (2)	12 (21)
ICM 4	Ectoderm	21	0 (0)	20 (95)	1 (5)
	Endoderm	41	37 (90)	2 (5)	2 (5)
ICM 5	Ectoderm	20	0 (0)	16 (80)	4 (20)
	Endoderm	25	17 (68)	5 (20)	3 (12)
ICM 6	Ectoderm	27	0 (0)	24 (89)	3 (11)
	Endoderm	44	32 (73)	1 (2)	11 (25)
Total	Ectoderm	151	0 (0)	140 (93)	11 (7)
	Endoderm	252	211 (84)	11 (4)	30 (12)

in dissociated endodermal fragments is probably due to contamination since none were seen in the endoderm of ICM 1 (Table 5). Equivocal or I cells constituted approximately 10 % of the total, and were found in dissociates of both tissues.

DISCUSSION

Providing vigorous mechanical dissociation of 4·5-day *p.c.* ICMs is avoided, the majority of separated cells may be classified as R or S in morphology. Furthermore, if microsurgically isolated primitive endoderm and primitive

ectoderm are dissociated separately, R cells can be assigned to the former and S cells to the latter tissue. The present results demonstrate that unequivocal R and S cells can also be distinguished by their patterns of colonization of host blastocysts from the blastocoelic cavity, providing chimaerism is not confined to the placenta and adjacent visceral yolk-sac residue. Thus R cells colonize the endoderm of the yolk sac, but are not found in its adjacent mesoderm or in the foetus, including principally endodermal derivatives of the latter such as the gut, liver and lungs (type A chimaerism). In contrast, unequivocal S cells colonize the mesodermal component of the yolk-sac and/or foetal structures, but their progeny were never detected in the yolk-sac endoderm (type B chimaerism). In no case was chimaerism detected in both layers of the visceral yolk sac, even in experiments using single donor cells of intermediate morphology. Furthermore, particularly in multiple cell injection experiments, donor contributions to many fractions in type B chimaeras were too high to be attributed to colonization of haemopoietic tissue alone (Table 4). In 4 of the 37 chimaeras analysed in this study the tissue distribution of donor cells could not be established, because they were detected only in the placenta and/or the unseparated residue of the yolk sac (chimaeras 9 and 10 in Table 2, and 31 and 37 in Table 4).

In an earlier study, injection of crudely dissected pieces of primitive endoderm tissue into blastocysts typically yielded yolk-sac and placental chimaerism only, while the majority of corresponding ectodermal fragments produced chimaerism in the foetus as well (Gardner & Papaioannou, 1975). These results accord with the findings reported in this paper. The present results demonstrate that morphological differentiation of the ICM in the late blastocyst into two populations of cells is accompanied by the acquisition of distinct cell states, as judged by the criterion of behaviour of the cells following transplantation. We believe, furthermore, that they provide an accurate description of the normal lineage of primitive endoderm and ectoderm cells. If this is the case, the former cells give rise to the extra-embryonic endoderm of the conceptus, and the latter to the entire foetus plus extra-embryonic mesoderm. Experiments on ectopic grafting of germ layers isolated from early post-implantation mouse and rat embryos support this conclusion (Diwan & Stevens, 1976; Skreb, Svajger & Levak-Svajger, 1976). Several workers have indeed adopted the terms hypoblast and epiblast rather than primitive endoderm and primitive ectoderm in recognition of the similarity in fate of early endoderm and ectoderm in the mouse and avian embryo (e.g. McLaren, 1976; Hogan & Tilly, 1977). However, confirmation of these proposed lineages in the mouse requires development of a cell marker that will enable investigation of the distribution of donor cells in constituent tissues of the chorio-allantoic placentae of type A and B chimaeras, and extension of analysis to the parietal or distal layer of the extra-embryonic endoderm.

Pedersen, Spindle & Wiley (1977) have demonstrated that giant ICMs

obtained by morula aggregation are able to form a new envelope of endoderm in culture after immunosurgical destruction of the original layer. They conclude that primitive ectoderm cells are capable of regenerating primitive endoderm, and are therefore not determined until later in development. However, they neither exclude nor entertain the alternative possibility that they may be witnessing formation of the definitive embryonic endoderm. Similar experiments carried out on ICMs from standard blastocysts provided no evidence of formation of a second endoderm layer, though the isolated primitive ectoderm fragments continued to grow and produced a variety of cell types (Hogan & Tilly, 1977). The present findings seem to require that the two types of 4·5-day ICM cells are relatively stably differentiated with respect to properties that either ensure their very precise partitioning in host embryos, or allow proliferation only of those that happen to reach the appropriate location. Clearly, it will be necessary to carry out short-term injection experiments to discriminate between these alternatives.

Finally, it is important to emphasize that donor cells were placed either in, or at least close to, their normal location in host blastocysts which were then returned to the appropriate *in vivo* environment. These experiments do not exclude the possibility that the two cell states may not be maintained in physiologically abnormal situations such as ectopic grafts and *in vitro* culture.

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