J. Embryol. exp. Morph. **80**, 225–240 (1984)
Printed in Great Britain © *The Company of Biologists Limited 1984*

Heterogeneous differentiation of external cells in individual isolated early mouse inner cell masses in culture

By J. NICHOLS AND R. L. GARDNER

Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford, OX1 3RE, U.K.

SUMMARY

Inner cell masses (ICMs) were isolated from early blastocysts by immunosurgery and incubated in a dense suspension of melanin granules for 3 h after 21 h in culture. The majority of such labelled ICMs subsequently formed outgrowths *in vitro* in which either giant cells or small solitary cells contained melanin granules. However, a substantial minority produced outgrowths in which both types of cell were unequivocally labelled. Labelled cells appeared from the results of control experiments to have originated within the external layer of the ICM. The giant cells were indistinguishable morphologically from those formed by authentic trophectodermal tissue. The small cells were identified as belonging to the extraembryonic endodermal lineage on the basis of their distribution in host conceptuses following injection into blastocysts. These findings support the conclusion reached in previous studies that early ICM cells can engage in trophectodermal differentiation under certain conditions. In addition, by providing evidence that both trophectoderm and endoderm cells can differentiate from the outer layer of the same ICM, they argue that loss of cellular lability is not coordinated throughout this tissue. Heterogeneity in the differentiation of external cells may depend on differences in both the stage of the mitotic cycle and the number of such cycles that they have completed since fertilization.

Finally, cell number in isolated early ICMs was found to increase approximately two-fold during the first 24 h of culture in the present experiments. This contrasts with the results of previous experiments in which cell number either increased more modestly or failed to do so altogether.

INTRODUCTION

Inner cell masses (ICMs) can only be isolated cleanly from 4th day mouse blastocysts by microsurgery once the embryos have attained a well-expanded state (Gardner & Johnson, 1972). Their constituent cells appear to have lost the capacity to form trophectoderm by this juncture (Rossant, 1975*a*,*b*). The development of immunosurgery by Solter & Knowles (1975) has enabled ICMs to be isolated routinely from earlier blastocysts. A substantial proportion of ICMs recovered immunosurgically from early cavitating blastocysts have been found to reorganize into structures which resemble blastocysts or trophectodermal vesicles following a period of 24–36 h in culture (Handyside, 1978; Hogan

& Tilly, 1978; Rossant & Tamura-Lis, 1979; Spindle, 1978). Evidence that such structures do indeed contain trophectodermal tissue was based initially on their giving rise to cells with the morphological characteristics of trophoblastic giant cells *in vitro* and in ectopic grafts (Handyside, 1978; Hogan & Tilly, 1978; Spindle, 1978). Subsequently, more compelling evidence was provided by Rossant & Tamura-Lis (1979) who showed that these blastocyst-like vesicles can induce decidua and form normal-looking postimplantation embryos *in utero*. These studies have led to the conclusion that ICMs of early blastocysts contain cells which have retained the capacity to undergo trophectodermal differentiation. The alternative possibility that residual trophectoderm cells which have escaped complement-mediated lysis (see Harlow & Quinn, 1979; Gardner, 1981) can account for this phenomenon seems unlikely in view of the extensive controls undertaken in one of the above studies (Handyside, 1978; also see Handyside & Barton, 1977).

Some of the ICMs from early blastocysts appear to form an external layer of endoderm rather than trophectoderm during culture *in vitro* (Handyside, 1978; Hogan & Tilly, 1978; Rossant & Tamura-Lis, 1979). Outgrowth of small solitary cells may accompany, or occur instead of, the differentiation of an endodermal layer (Handyside, 1978). The remaining ICMs either fail to exhibit any further morphological differentiation or form irregular masses containing fluid-accumulating cells which Handyside (1978) has termed non-integrated forms. The response to *in vitro* culture of ICMs isolated immunosurgically from expanded 4th day blastocysts is consistent with earlier results in which microsurgery was used. Thus, regeneration of a trophectodermal layer by such ICMs is exceptional, formation of an outer endodermal layer taking place in nearly all specimens that exhibited further morphological development (Handyside, 1978; Hogan & Tilly, 1978; Rossant & Tamura-Lis, 1979; Spindle, 1978). Therefore, the pattern of cellular differentiation in ICMs evidently changes according to their maturity at isolation. One obvious factor that might underlie such a change is the number of mitotic cycles that the cells have completed by the stage at which the ICMs are isolated (Gardner, 1983). In view of the fact that cell cycles become markedly asynchronous well before blastulation in the mouse (Kelly, Mulnard & Graham, 1978; Smith & McLaren, 1977), one might anticipate that the changeover from trophectodermal to endodermal differentiation by isolated ICMs would be gradual rather than abrupt. However, Handyside (1978) specifically noted that he observed no cases in which individual isolated ICMs yielded both trophectoderm and endoderm cells. Hogan & Tilly (1978). in contrast, reported that isolated ICMs giving rise to an external endoderm layer tended to form egg-cylinder-like structures which appeared to contain extraembryonic ectoderm, a trophectoderm derivative (see Gardner, 1983), following more prolonged culture *in vitro*. Moreover, when disrupted by pipetting, such structures consistently gave rise to outgrowths containing trophoblast-like giant cells. These findings are compatible with the notion that both trophectoderm and

endoderm can differentiate from cells within individual isolated ICMs. However, they do not provide conclusive proof that this is so, because incomplete lysis of the original trophectoderm was not excluded altogether in this investigation.

The principal aim of the present investigation was to examine more rigorously whether both trophectoderm and extraembryonic endoderm cells can indeed differentiate within individual isolated ICMs *in vitro*. In addition, cell counts were undertaken in order to determine the extent to which growth of ICM tissue is affected by culture during the first 24 h following isolation. According to Handyside (1978) and Spindle (1978), mean cell number in isolated ICMs does not increase during 24–48 h in culture; it may even exhibit a slight decrease. However, this might be due to enhanced cell death under certain culture conditions rather than to the absence of cell proliferation since, according to the limited data of Rossant & Tamura-Lis (1979), a modest increase in cell number may occur during the first 24 h of culture. Regardless of the explanation for these apparently discrepant findings, growth of the ICM is evidently impaired in these circumstances since it would be expected to show at least a doubling in cell number if left for a similar interval in its normal environment *in vivo* (Copp, 1978). A better understanding of the way in which growth of this tissue is altered following isolation may help to elucidate the factors which determine the particular pattern of differentiation which it exhibits *in vitro*.

MATERIALS AND METHODS

Mice

All mice, except those used to provide suspensions of melanin granules, belonged to one or other of two closed colonies which had been derived from the random-bred PO (Pathology, Oxford) albino strain. The two stocks were homozygous for different alleles at the locus coding for glucose phosphate isomerase (GPI), one being *Gpi-1*^{a}/*Gpi-1*^{a} and the other *Gpi-1*^{b}/*Gpi-1*^{b}. They were maintained on a 24 h light–dark cycle in which the period of darkness extended from 19.00–07.00 h, and were provided with food and water *ad libitum*. Mature females were examined externally (Champlin, Dorr & Gates, 1973) and those that appeared to be in oestrus were placed overnight with males. Matings were arranged so that donor embryos would be *Gpi-1*^{b}/*Gpi-1*^{b} and host embryos *Gpi-1*^{a}/*Gpi-1*^{a} in the single series of experiments in which cells from cultured ICMs were injected into blastocysts. In addition, several *Gpi-1*^{a}/*Gpi-1*^{a} females were mated with vasectomized males to provide uterine foster mothers for the injected blastocysts. Matings between *Gpi-1*^{a}/*Gpi-1*^{a} individuals were used to provide the embryos used in all other experiments. Females exhibiting vaginal plugs on the morning after they had been placed with males were presumed to be in the first day of pregnancy or pseudopregnancy.

Media

A modification of the PB1 medium of Whittingham & Wales (1969) supplemented with 10 % (v/v) foetal calf serum (Gardner, 1982) was used for the recovery, manipulation and storage of embryos at room temperature. Unless stated otherwise, PB1 refers throughout the text to the serum-supplemented form of the modified medium. Culture of intact embryos and isolated ICMs was carried out under liquid paraffin (Boots Pure Drug Co., U.K.) in drops of modified α medium (Stanners, Eliceiri & Green, 1971) which was invariably supplemented with 10 % (v/v) foetal calf serum. The cultures were maintained at 37 °C in a humidified atmosphere of 5 % CO_2 in air. Isolated ICMs that had been exposed to pronase were cultured under similar conditions in OC medium (table 6.5 in Biggers, Whitten & Whittingham, 1971) from which calcium salts had been omitted in order to promote their decompaction.

Embryos

Host blastocysts for the cell injection experiments were recovered from 14.30 h on the 4th day of pregnancy. Otherwise, embryos were recovered somewhat earlier on the 4th day, beginning at approximately 11.30 h. After pooling, embryos destined for culture were placed in a small drop of acidified Tyrode's saline (Nicholas & Hall, 1942; Nicolson, Yanagimachi & Yanagimachi, 1975) which was neutralized by addition of a much larger volume of PB1 as soon as dissolution of *zonae pellucidae* was complete. The denuded embryos were then cultured for 1 h prior to detailed classification. Only those embryos classified as early cavitating blastocysts in which the volume of the blastocoele was equal to or less than that of the ICM were selected for immunosurgery and/or culture.

Immunosurgery

Basically, the procedure described by Solter & Knowles (1975) was adopted but with the introduction of certain modifications. Thus, a rabbit antiserum was used which had been raised against mouse foetal tissue rather than adult spleen cells. In addition, rat serum was employed as a source of complement because, unlike guinea pig serum, it proved consistently to be non-toxic (Spielmann, Jacob-Muller & Beckord, 1980; R. L. Gardner, unpublished observations). Both the rabbit and rat sera were stored at −70 °C as 0·05 ml aliquots to which 0·45 ml of PB1 minus serum was added immediately before use. Embryos were first incubated in the diluted antiserum at 37 °C for 15 or 30 min and then, after three rinses in PB1, for an additional 8 min in the diluted rat serum. Following a further rinse in PB1, the embryos were cultured in α medium for approximately 45 min. Finally, each ICM was freed from investing trophectodermal debris with the aid of a glass micropipette which had been heat polished to an appropriate tip diameter.

Cell counts on ICMs

The method described by Tarkowski (1966) was found to work reliably only for freshly isolated ICMs (time zero counts). Insufficient spreading of nuclei for accurate counting was observed consistently when it was applied to ICMs which had been cultured. Following investigation of various other ways of determining their cell number, cultured ICMs were treated as follows. They were incubated first at 4 °C in a solution of pronase (Calbiochem. grade B) made up in Dulbecco A phosphate-buffered saline (PBS, Oxoid) for 8–10 min. A 0·25 % (w/v) solution of the enzyme preparation was used for most of the time 0 and all the 6 and 12 h cultured ICMs, and a 0·5 % solution for the 24 h cultured specimens. Thereafter, the ICMs were rinsed in calcium-free OC medium and then incubated at 37 °C in pre-equilibrated drops of this medium in bacteriological grade plastic dishes (Sterilin, U.K.). The drops were inspected at intervals beginning at 20 min of culture, and ICMs transferred individually to separate hanging drops in a manipulation chamber (Puliv, Leitz) once they had clearly decompacted. Each ICM was then carefully opened up with a pair of round-tipped siliconized glass needles held in Leitz micromanipulators so that the total number of cells could be counted precisely.

Labelling of blastocysts and ICMs with melanin granules

Iris epithelia were dissected from the eyes of groups of four adult pigmented mice of variable genetic constitution. They were teased apart repeatedly in α medium with the aid of watch-makers forceps until a dense suspension of melanin granules had been released. The suspension was allowed to stand for 2 h at 4 °C to enable sedimentation of debris and larger particles, and then transferred to a siliconized (Repelcote, Hopkin & Williams, U.K.) centrifuge tube in which it was made up to approximately 8 ml by addition of more α medium. The granules were sedimented by centrifugation at 4300 r.p.m. for 25 min, being re-suspended thereafter in roughly 0·5 ml of medium by vigorous agitation. Next, small volumes of the suspension were added to drops of α medium in bacteriological dishes which contained blastocysts or ICMs, and the dishes cultured at 37 °C. Intact blastocysts were exposed to melanin granules immediately after the 1 h culture period that followed removal of their *zonae*. Isolated ICMs were cultured for approximately 21 h prior to exposure to the particles. The contents of the drops were re-suspended at 1 h and again at 2 h of incubation in order to encourage extensive surface labelling of the embryonic material. After 3 h the blastocysts or ICMs were removed from the drops and rinsed free of unattached granules by repeated sedimentation through fresh α medium. Thereafter, some of the ICMs were processed for serial sectioning, the remainder being cultured individually in microdrops in tissue culture dishes (Falcon, Oxnard, U.S.A.) for up to 1 week. Some of the labelled blastocysts were cultured similarly, either directly or following immunosurgery. All cultures were left undisturbed for

approximately 40 h, and thereafter inspected twice daily in a Leitz Diavert microscope. The remaining labelled blastocysts were processed for serial sectioning.

Histology and analysis of histological preparations

ICMs and intact blastocysts which were to be serially sectioned following labelling with melanin granules were fixed in 2·5 % glutaraldehyde (B.D.H., U.K.) in 0·2 M-phosphate buffer for 10 min at room temperature, rinsed in fresh buffer, dehydrated in ethanol and then embedded in resin (Emix, Emscope, U.K.). The ICMs were sectioned at 1 μm and the blastocysts at 2 μm on a Huxley ultramicrotome, the sections being stained with 1 % toluidine blue in 1 % borax prior to examination in a Zeiss RA microscope.

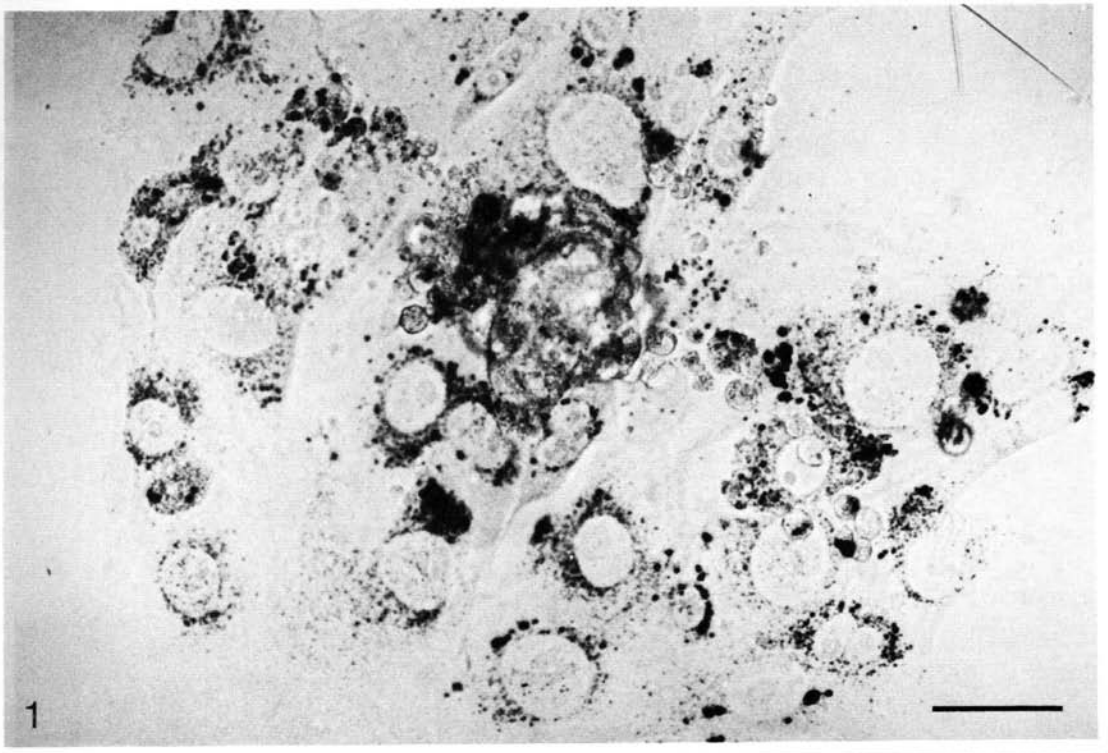
The distribution of melanin granules in sections of ICMs was invariably assessed at a magnification of ×1000. A magnification of either ×400 or ×1000 was used for reconstructing the sectioned blastocysts on glass plates with the aid of a Zeiss drawing tube. Cell boundaries that are roughly parallel to the plane of sectioning could not be resolved. Hence, an external cell was scored as labelled only if melanin granules occurred within or attached to its surface in sections which included the nucleus or, if division was in progress, a cluster of chromosomes.

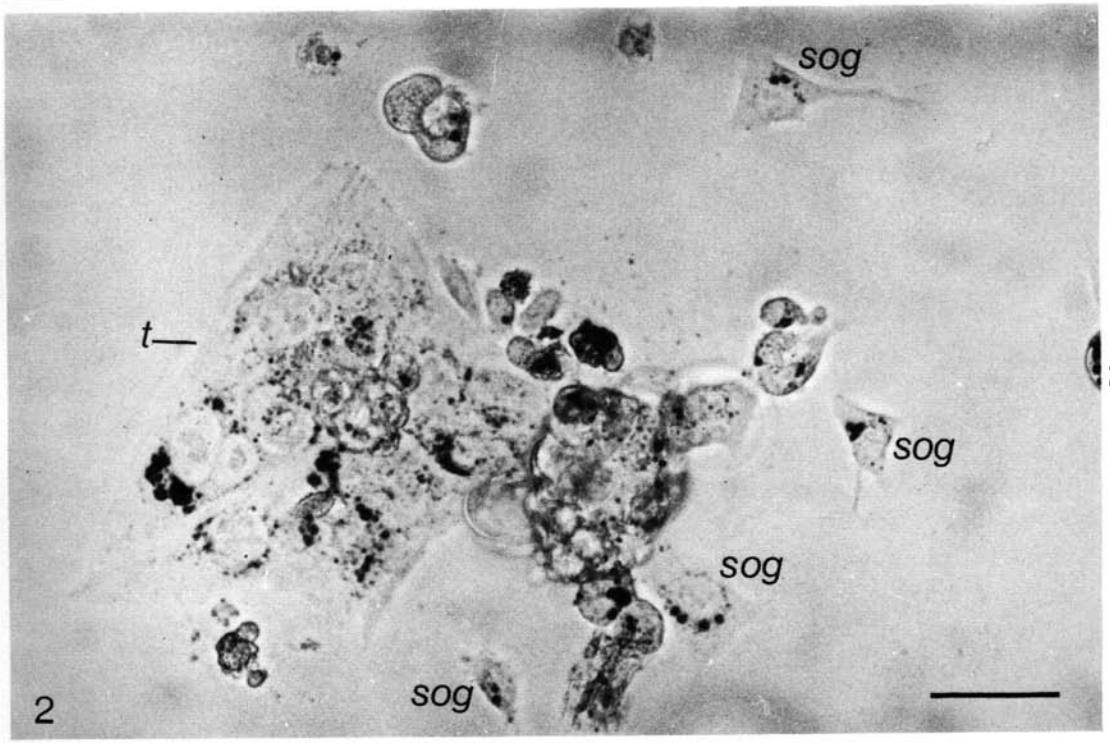
Production and analysis of chimaeras

Microdrops containing individual *Gpi-1*^{b}/*Gpi-1*^{b} ICMs that had been cultured for 3 days were inspected for outgrowth of small irregularly shaped solitary cells. Drops containing several such cells were cleared of all other cells and tissue. As much fluid as possible was then removed from them and replaced twice with 0·1 % trypsin (Difco, 1/250) in calcium-/magnesium-free Tyrode's saline. After approximately 5 min exposure to the enzyme solution at room temperature, the small outgrowth cells were released by gentle pipetting and transferred to hanging drops in manipulation chambers. Either three or five cells were injected into each *Gpi-1*^{a}/*Gpi-1*^{a} host blastocyst as described previously (Gardner, 1978, 1982). The injected blastocysts were transferred to the uteri of recipient females late in the afternoon of the third day of pseudopregnancy. Recipients were killed approximately 9 days later and all conceptuses dissected into various tissue fractions (see Gardner, 1982, for details). Electrophoresis of frozen and thawed tissue samples was carried out on Titan III plates (Helena Laboratories) according to the procedure of McLaren & Buehr (1981), except that 0·025 % rather than 0·09 % tris-glycine was used as the bridge buffer. Following staining by the

Fig. 1. Labelled blastocyst after 4 days in culture showing association of melanin granules with all nuclei in the outgrowing sheet of trophoblast cells. (×350).

Fig. 2. Labelled ICM after 3 days in culture showing presence of melanin granules in both small outgrowth cells (sog) and incipient giant cells (t). (×350).





Figs 1 and 2

method of Eicher & Washburn (1978), the plates were scanned with a Gelman DCD-16 densitometer to determine the relative proportions of donor and host allozyme activities of GPI.

RESULTS

Control labelling experiments

All external cells were unequivocally labelled with melanin granules in 10 out of 11 sectioned blastocysts which were suitable for serial reconstruction. All such cells except one, a metaphase, were unequivocally labelled in the remaining embryo.

Fifty-one out of 53 blastocysts that were cultured intact following labelling with melanin granules gave rise to conspicuous coherent outgrowths of giant cells. Melanin granules could readily be discerned throughout the outgrowths in 47 cases (Fig. 1), but appeared to be somewhat more restricted in distribution in the remaining four.

Ninety-eight ICMs were isolated satisfactorily from a further 103 blastocysts which were subjected to immunosurgery after labelling. One or more giant cells outgrew from 39 of these ICMs during subsequent culture and, except in one case, all such cells were unlabelled. A single unequivocal clump of melanin granules was observed in the sheet of giant cells formed by the exceptional ICM. Three labelled small outgrowth cells were produced by one of the remaining 59 ICMs which did not yield any giant cells.

Thirty isolated ICMs that appeared to have been relatively uniformly and densely labelled with melanin granules were processed for serial sectioning. The granules were observed in the cytoplasm and/or attached to the free surfaces of external cells only in all cases.

Culture of labelled ICMs

A completely independent series of experiments was carried out by each author in which ICMs were encouraged to outgrow in culture after labelling with melanin granules. As shown in Table 1, a high proportion of the ICMs exhibited outgrowth of unequivocally labelled cells in both series. Melanin granules were confined either to giant cells or small outgrowth cells in the majority of cases. However, a substantial minority of individual ICMs produced both types of labelled cell (Fig. 2). Furthermore, despite a significant difference between the two sets of experiments in the proportion of ICMs yielding labelled cells of one or the other type only, there was a remarkably close agreement between them regarding the frequency with which both types of cell were labelled (Table 1). The small cells typically appeared at 2 to 3 days of culture and had usually begun to round up and deteriorate before unequivocal giant cells had formed.

Each ICM was assigned to one of three morphological categories when it was

Table 1. *Development of labelled ICMs in culture*

		Classification of ICMs				
	Experiment No.	No. showing outgrowth of labelled small cells only	No. showing outgrowth of labelled giant cells only	No. showing outgrowth of labelled small cells and labelled giant cells	No. showing outgrowth of unlabelled cells only	No. showing no outgrowth (No. degenerating)
Series 1 (R.L.G.)	1	7	3	4	1	2(1)
	2	5	11	2	2	2(1)
	3	4	6	5	4	0(0)
	4	9	9	4	3	2(2)
	TOTAL	25	29	15	10	6(4)
	%	29·4	34·1	17·6	11·8	7·1(4·7)
Series 2 (J.N.)	5	7	8	1	0	2(1)
	6	3	1	3	3	4(3)
	7	23	9	7	0	5(2)
	8	15	4	6	0	11(9)
	TOTAL	48	22	17	3	22(15)
	%	42·8	19·6	15·2	2·7	19·6(13·4)

first placed in culture after labelling. As shown in Table 2, ICMs belonging to all three classes could give rise to outgrowths in which both small cells and giant cells were labelled. Nevertheless, vesiculated and solid structures most commonly formed outgrowths in which only giant and small cells, respectively, were labelled.

Table 2. *Relationship between initial morphology of ICMs after labelling and pattern of differentiation of labelled cells in resulting outgrowths*

		Type(s) of labelled cells in outgrowths		
	Morphology of ICM	No. (%) ICMs with labelled small cells only	No. (%) ICMs with labelled giant cells only	No. (%) ICMs with labelled small cells and labelled giant cells
Series 1*	Vesiculated	5(15)	22(67)	6(18)
	Solid	8(57)	2(14)	4(29)
	Non-integrated	12(54)	5(23)	5(23)
Series 2*	Vesiculated	7(23)	18(58)	6(19)
	Solid	16(94)	0(0)	1(6)
	Non-integrated	25(64)	4(10)	10(26)

* see Table 1.

Table 3. *Donor cell contribution to the parietal endoderm in chimaeric conceptuses obtained following injection of* Gpi-1^b/Gpi-1^b *small outgrowth cells into* Gpi-1^a/Gpi-1^a *blastocysts*

Conceptus No.*	No. of donor cells injected/blastocyst	% Donor GPI contribution
R1/1	3	4·2
R1/2	3	22·6
R1/3	3	1·8
R1/4	3	3·7
R1/5	3	4·9
R3/3	3	15·2
R3/4	3	6·8
R3/5	3	5·9
R4/2	3	7·9
R4/3	3	9·8
R4/4	3	7·4
R4/5	3	7·5
R4/7	5	11·6
R4/8	5	11·8
R4/9	5	18·0

* A further four conceptuses were non-chimaeric. Additonal fractions analysed were placenta, trophoblastic giant cells, endoderm of visceral yolk sac, mesoderm of visceral yolk sac, and foetus plus amnion and umbilical cord. These exhibited host allozyme activity only in all cases.

Injection of small outgrowth cells into blastocysts

Three cells were injected into each of 20 blastocysts, and five cells into each of a further four. Fifteen of the 19 normal conceptuses obtained from the transplanted blastocysts were chimaeric. Chimaerism was detectable in the parietal endoderm only in all cases and, though variable, the donor contribution to this tissue was without exception considerably less than that of the host (Table 3).

Cell counts on isolated ICMs

Table 4 shows the mean cell number in freshly isolated ICMs and in ICMs that had been cultured for different periods following isolation. It is clear that cell number varied considerably even at time 0, despite the precautions that were taken to limit the heterogeneity in stage of development of blastocysts used in the present investigations. It is also evident that mean cell number approximately doubles during culture of ICMs for 24 h on a bacteriological surface, the largest increment occurring during the first 6 h. Finally, Table 5 shows the relationship between cell number and morphology for the 24 h cultured ICMs which were

Table 4. *Cell counts on decompacted ICMs*

Time in culture after immunosurgery (hours)	0	+6	+12	+24
No. of ICMs sampled	47	35	44	51*
Mean cell No. (±S.D.)	15·8 (± 6·5)	24·8 (± 6·0)	26·6 (± 7·8)	29·9 (± 12·2)

* Thirty five of these ICMs were classified morphologically before decompaction for cell counts, as shown in Table 5.

Table 5. *Relationship between morphology and cell number in isolated ICMs cultured for 24 h*

	Morphology of ICMs		
	Solid	Vesiculated	Non-integrated
No. of ICMs scored	10	7	18
Mean cell No. (± S.D.)	43 (± 16·0)*	23·9 (± 7·8)	22·9 (± 9·2)

* Mean cell number in solid structures is significantly different ($P < 0{\cdot}001$) from that in vesiculated and non-integrated forms. Vesiculated and non-integrated structures do not differ significantly in cell number.

classified prior to decompaction. The mean cell number was significantly higher in the solid structures than in the vesiculated or non-integrated forms.

DISCUSSION

If intact early cavitating blastocysts are exposed to a suspension of melanin granules for 3 h all their external cells typically become labelled with these particles. Furthermore, melanin granules can also be seen in most if not all giant cells that are formed if labelled blastocysts are allowed to outgrow subsequently *in vitro*. Morphologically indistinguishable giant cells develop in a substantial proportion of outgrowths formed by the residual clusters of viable cells that are recovered when such blastocysts are subjected to immunosurgery after labelling. However, only one giant cell was found to be labelled in 39 outgrowths from immunosurgically-treated blastocysts producing such cells. The exceptional cell may have been a trophectodermal cell which had resisted lysis (Harlow & Quinn, 1979). An alternative possibility which would seem to explain more readily the occurrence of labelled small cells in a further outgrowth, is that the granules were acquired by ICM-derived cells from trophectodermal debris that had not been removed completely following immunosurgery. Nevertheless, the fact that giant cells obtained following exposure of labelled blastocysts to immunosurgery were typically unlabelled argues that, in agreement with earlier claims (Handyside, 1978; Hogan & Tilly, 1978; Rossant & Tamura-Lis, 1979; Spindle, 1978), they were of ICM rather than trophectoderm origin. The trophectoderm cells that give rise to the extraembryonic ectoderm (Copp, 1979; Gardner, 1983) might escape both labelling and lysis in early blastocysts if internalized shortly after blastulation. However, giant cell production by immunosurgically-isolated ICMs would be expected to increase rather than decline in frequency with maturation of the blastocyst (Handyside, 1978; Rossant & Tamura-Lis, 1979; Spindle, 1978) if it was due to the presence of such cells.

When freshly isolated from 4th day blastocysts, ICM tissue does not readily phagocytose melanin granules (Gardner, 1975*a*). However, following culture of the ICMs for 21 h, substantial uptake of granules was found to occur during a 3 h period. Furthermore, labelling with these particles was confined exclusively to external cells under these conditions. Hence cells containing melanin granules that appeared during subsequent outgrowth must either have been on the outside of ICMs during labelling or have acquired their granules secondarily from external cells. No evidence in support of the most unlikely possibility of the regular transfer of melanin granules between cells was obtained in either the present or previous experiments (Gardner, 1975*b*, 1977; Copp, 1979), or in very recent studies in which cells labelled with these particles could be distinguished from unlabelled cells by means of a second independent marker (Gardner, 1984).

Unequivocally labelled cells outgrew from a high proportion of ICMs that were cultured on a substratum to which they could adhere following labelling.

The labelled cells could readily be assigned to one or other of two distinct classes on the basis of size, shape, and proximity to other cells. Cells belonging to one or other class only were labelled in the majority of outgrowths, although the two series of experiments differed markedly in the frequency with which labelling was restricted to small versus giant cells (Table 1). This disparity may reflect subtle differences between authors in choice of blastocysts and handling of ICMs. A comparison of records suggested, for example, that R.L.G. used a higher proportion of minimally cavitated blastocysts than J.N.. Nevertheless, a substantial minority of ICMs in each series of experiments yielded outgrowths in which both types of cell were labelled. The obvious conclusion to be drawn from this interesting finding is that external cells of individual cultured ICMs isolated from early blastocysts can pursue different developmental pathways. One pathway leads to the differentiation of characteristic uni- or bi-nucleate giant cells that are indistinguishable from those produced by trophectodermal vesicles (Ansell, 1975; Ansell & Snow, 1975; Sherman, 1975; Sherman & Salomon, 1975). The other results in formation of smaller irregularly shaped cells that resemble morphologically the early parietal endoderm cells of the implanting blastocyst (see Enders, Given & Schlafke, 1978). The extraembryonic endodermal nature of small outgrowth cells is evident from their distribution in host conceptuses following injection into blastocysts. Thus, colonization of the parietal endoderm only was detected (Table 3), as is typically the case in chimaeras produced by injecting unequivocal extraembryonic endoderm cells from periimplantation embryos into blastocysts (Gardner, 1982). It is likely that external cells of isolated ICMs have already embarked on trophectodermal or endodermal differentiation at the time of labelling. However, the present experiments do not exclude the possibility that individual cells are still able to produce both types of descendants at this juncture.

One might expect the pattern of differentiation of external cells to be reflected in the morphology of the isolated ICMs following culture for 24 h on a bacteriological surface. Thus, formation of mini-blastocysts or vesicles should result from trophectodermal differentiation while retention of a regular solid appearance might be expected to accompany endodermal differentiation of outer cells. On this basis, non-integrated forms would be anticipated in cases in which the external layer exhibited differentiation of both endoderm and trophectoderm cells. In practice, however, several factors tend to complicate attempts to define the relationship between the morphology of ICMs after labelling and their subsequent pattern of outgrowth of labelled cells. First, the incidence of vesiculated ICMs is likely to be underestimated since those that differentiate more slowly or contract during labelling or handling thereafter will be scored as either solid or non-integrated structures. Second, labelled cells of both types will be missed if they fail to outgrow from the central mass of cells that persists in most cases. Third, division of labelled cells may yield outgrowth of only equivocally labelled or unlabelled progeny.

Clearly, ICMs that are vesiculated after labelling most commonly yield outgrowths in which giant cells only are labelled while those that remain solid mainly form outgrowths in which only small cells are labelled. Nevertheless, these two morphological categories of ICM can also produce outgrowths containing both types of labelled cells. It is also evident from Table 2 that non-integrated forms give rise more frequently to outgrowths in which labelling is confined to small cells only than those in which it is present in both types of cell. The spatial relationship between cells that undergo endodermal versus trophectodermal differentiation is likely to be as important as their proportions in accounting for the variable morphology of ICMs whose external cells are found on subsequent outgrowth to have behaved in a mosaic fashion.

Since ICMs exhibiting a mixed pattern of differentiation occurred in all eight series of experiments (Table 1) this behaviour is more readily attributable to heterogeneity of the cells than the culture environment. An obvious source of such heterogeneity is the asynchrony in cell cycles that is already manifest in early cleavage (Kelly, Mulnard & Graham, 1978) and becomes very marked following blastocyst formation (Smith & McLaren, 1977). The possibility that this is the decisive factor in determining the differential response of external ICM cells in culture is currently being explored.

It is clear that under the conditions employed in the present experiments, ICM cells do continue to proliferate during the critical 24 h in culture during which differentiation of external cells takes place. However, the extent to which external cells participate in this approximate doubling in total cell number has yet to be explored (Gardner, 1983).

In conclusion, the present findings support those of previous studies in suggesting that some ICM cells in the early blastocyst retain the capacity for trophectodermal differentiation under certain conditions. In addition, they provide evidence that loss of this option can be markedly asynchronous among the cells of a single ICM. The stage in maturation of the blastocyst at which lability of ICM cells is finally lost has yet to be established. Finally, it should perhaps be noted that neither the present nor previous investigations provide an answer to the question whether the ICM normally contributes cells to the trophectoderm in the early blastocyst.

We wish to thank Dr Rosa Beddington, Dr David Cockroft and Dr Christopher Graham for advice, and Mrs Marilyn Carey, Miss Judy Green, Mr Philip Thornton-Evison and Mrs Jo Williamson for help in preparing the manuscript. The work was supported by the Imperial Cancer Research Fund and The Royal Society.

REFERENCES

ANSELL, J. D. (1975). The differentiation and development of mouse trophoblast. In *The Early Development of Mammals*; *2nd Symposium of Brit. Soc. Dev. Biol.* (eds M. Balls & A. E. Wild), pp. 133–144. Cambridge: Cambridge University Press.

ANSELL, J. D. & SNOW, M. H. L. (1975). The development of trophoblast *in vitro* from blastocysts containing varying amounts of inner cell mass. *J. Embryol. exp. Morph.* **33**, 177–185.

BIGGERS, J. D., WHITTEN, W. K. & WHITTINGHAM, D. G. (1971). The culture of mouse embryos *in vitro*. In *Methods in Mammalian Embryology* (ed. J. D. Daniel), pp. 86–116. San Francisco: Freeman.

CHAMPLIN, A. K., DORR, D. L. & GATES, A. H. (1973). Determining the stage of the estrous cycle in the mouse by the appearance of the vagina. *Biol. Reprod.* **8**, 491–494.

COPP, A. J. (1978). Interaction between inner cell mass and trophectoderm of the mouse blastocyst. I. A study of cellular proliferation. *J. Embryol. exp. Morph.* **48**, 109–125.

COPP, A. J. (1979). Interaction between inner cell mass and trophectoderm of the mouse blastocyst. II. The fate of the polar trophectoderm. *J. Embryol. exp. Morph.* **51**, 109–120.

EICHER, E. M. & WASHBURN, L. L. (1978). Assignment of genes to regions of mouse chromosomes. *Proc. natn. Acad. Sci., U.S.A.* **75**, 946–950.

ENDERS, A. C., GIVEN, R. L. & SCHLAFKE, S. (1978). Differentiation and migration of endoderm in the rat and mouse at implantation. *Anat. Rec.* **190**, 65–78.

GARDNER, R. L. (1975*a*). Origins and properties of trophoblast. In *Immunobiology of Trophoblast* (eds R. G. Edwards, C. W. S. Howe & M. H. Johnson), pp. 43–61. London and New York: Cambridge University Press.

GARDNER, R. L. (1975*b*). Analysis of determination and differentiation in the early mammalian embryo using intra- and inter-specific chimaeras. In *Developmental Biology of Reproduction, 33rd Symposium of the Society for Developmental Biology* (eds C. L. Markert & J. Papaconstantinou), pp. 207–238. New York: Academic Press.

GARDNER, R. L. (1977). The problem of intrauterine orientation of the implanting mouse blastocyst. *J. Anat.* **124**, 236 (Abstract).

GARDNER, R. L. (1978). Production of chimeras by injecting cells or tissue into the blastocyst. In *Methods in Mammalian Reproduction* (ed. J. C. Daniel), pp. 137–165. New York: Academic Press.

GARDNER, R. L. (1981). *In vivo* and *in vitro* studies on cell lineage and determination in the early mouse embryo. In *Cellular Controls in Differentiation* (eds C. W. Lloyd & D. A. Rees), pp. 257–278. New York: Academic Press.

GARDNER, R. L. (1982). Investigation of cell lineage and differentiation in the extraembryonic endoderm of the mouse embryo. *J. Embryol. exp. Morph.* **68**, 175–198.

GARDNER, R. L. (1983). Origin and differentiation of extra-embryonic tissues in the mouse. *International Review of Experimental Pathology Vol. 24* (eds G. W. Richter & M. A. Epstein), pp. 63–133. New York: Academic Press.

GARDNER, R. L. (1984). An *in situ* cell marker for clonal analysis of development of the extraembryonic endoderm in the mouse. *J. Embryol. exp. Morph.* **81**, 251–288.

GARDNER, R. L. & JOHNSON, M. H. (1972). An investigation of inner cell mass and trophoblast tissues following their isolation from the mouse blastocyst. *J. Embryol. exp. Morph.* **28**, 279–312.

HANDYSIDE, A. H. (1978). Time of commitment of inside cells isolated from preimplantation mouse embryos. *J. Embryol. exp. Morph.* **45**, 37–53.

HANDYSIDE, A. H. & BARTON, S. C. (1977). Evaluation of the technique of immunosurgery for the isolation of inner cell masses from mouse blastocysts. *J. Embryol. exp. Morph.* **37**, 217–226.

HARLOW, G. M. & QUINN, P. (1979). Isolation of inner cell masses from mouse blastocysts by immunosurgery or exposure to the calcium ionophore A23187. *Aust. J. biol. Sci.* **32**, 483–491.

HOGAN, B. & TILLY, R. (1978). *In vitro* development of inner cell masses isolated immunosurgically from mouse blastocysts. II. Inner cell masses from 3·5 to 4·0 day p.c. blastocysts. *J. Embryol. exp. Morph.* **45**, 107–121.

KELLY, S. J., MULNARD, J. G. & GRAHAM, C. F. (1978). Cell division and cell allocation in early mouse development. *J. Embryol. exp. Morph.* **48**, 37–51.

MCLAREN, A. & BUEHR, M. (1981). GPI expression in female germ cells of the mouse. *Genet. Res. Camb.* **37**, 303–309.

NICHOLAS, J. S. & HALL, B. V. (1942). Experiments on developing rats. II. The development of isolated blastomeres and fused eggs. *J. exp. Zool.* **90**, 441–459.

NICOLSON, G. L., YANAGIMACHI, R. & YANAGIMACHI, H. (1975). Ultrastructural localisation of lectin-binding sites on the zonae pellucidae and plasma membranes of mammalian eggs. *J. Cell Biol.* **66**, 263–274.

ROSSANT, J. (1975*a*). Investigation of the determinative state of the mouse inner cell mass. I. Aggregation of isolated inner cell masses with morulae. *J. Embryol. exp. Morph.* **33**, 979–990.

ROSSANT, J. (1975*b*). Investigation of the determinative state of the mouse inner cell mass. II. The fate of isolated inner cell masses transferred to the oviduct. *J. Embryol. exp. Morph.* **33**, 991–1001.

ROSSANT, J. & TAMURA-LIS, W. (1979). Potential of isolated mouse inner cell masses to form trophectoderm derivatives *in vivo*. *Devl Biol.* **70**, 255–261.

SHERMAN, M. I. (1975). The role of cell-cell interactions during early mouse embryogenesis. In *The Early Development of Mammals: 2nd Symposium of Brit. Soc. Dev. Biol.* (eds M. Balls & A. E. Wild), pp. 145–165. Cambridge: Cambridge University Press.

SHERMAN, M. I. & SALOMON, D. S. (1975). The relationship between the early mouse embryo and its environment. *Symp. Soc. devl Biol.* **33**, 277–309.

SMITH, R. & MCLAREN, A. (1977). Factors affecting the time of formation of the mouse blastocoel. *J. Embryol. exp. Morph.* **41**, 79–92.

SOLTER, D. & KNOWLES, B. B. (1975). Immunosurgery of mouse blastocyst. *Proc. natn. Acad. Sci., U.S.A.* **72**, 5099–5102.

SPIELMAN, H., JACOB-MULLER, U. & BECKORD, W. (1980). Immunosurgical studies on inner cell mass development in rat and mouse blastocysts before and during implantation *in vitro*. *J. Embryol. exp. Morph.* **60**, 255–269.

SPINDLE, A. I. (1978). Trophoblast regeneration by inner cell masses isolated from cultured mouse embryos. *J. exp. Zool.* **203**, 483–490.

STANNERS, C. P., ELICEIRI, G. L. & GREEN, H. (1971). Two types of ribosome in mouse-hamster hybrid cells. *Nature New Biol. Lond.* **230**, 52–54.

TARKOWSKI, A. K. (1966). An air-drying method for chromosome preparations from mouse eggs. *Cytogenetics* **5**, 394–400.

WHITTINGHAM, D. G. & WALES, R. G. (1969). Storage of two-cell mouse embryos in vitro. *Aust. J. biol. Sci.* **22**, 1065–1068.

(Accepted 17 November 1983)