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Regeneration of endoderm from primitive ectoderm in the mouse embryo: fact or artifact?

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SUMMARY

The capacity of immunosurgically (IS) treated inner cell masses (ICMs) *versus* microsurgically (MS) isolated primitive ectoderms from blastocysts recovered on the 5th day of gestation to regenerate an external layer of endoderm cells *in vitro* was investigated. While the majority of IS-treated ICMs regenerated such a layer, MS-isolated ectoderms seldom did so. Examination of the two types of tissue fragments revealed that IS-treated ICMs almost invariably retained viable endoderm cells whereas MS-isolated ectoderms did so only exceptionally. The endoderm was found to be more than one cell layer thick in ICMs from 5th day blastocysts, suggesting that some endoderm cells survive IS because they are protected from exposure to antiserum. Typing of the endoderm layer that regenerated following IS treatment of recombinant ICMs composed of genetically dissimilar endoderm and ectoderm provided direct evidence that it originated from residual endoderm cells rather than the underlying ectoderm. Finally, blastocyst injection experiments confirmed that IS-treated ICMs behave like a mixture of ectoderm and endoderm tissue *in vivo*, and provided no support for the view that cells of the original and regenerated endoderm differ in developmental potential. These findings challenge earlier conclusions concerning cell lineage and determination in the primitive ectoderm that were based on development *in vitro* of IS-treated ICMs from giant blastocysts.

INTRODUCTION

By the onset of implantation the inner cell mass (ICM) of the mouse blastocyst is clearly composed of two tissues, the primitive ectoderm and the primitive endoderm (Snell & Stevens, 1966; Nadijcka & Hillman, 1974). The primitive ectoderm comprises the deep ICM cells and the primitive endoderm the more superficial ones, including those bordering the blastocoelic cavity. Primitive endoderm cells differ ultrastructurally from primitive ectoderm cells in possessing a relatively conspicuous rough endoplasmic reticulum (RER) (Enders, 1971; Nadijcka & Hillman, 1974; Enders, Given & Schlafke, 1978). The fact that the two types of cell can also be reliably distinguished in the living state (Gardner & Rossant, 1979) has enabled critical comparison of their developmental potential *in vivo* by blastocyst injection. Chimaerism is confined to the visceral and/or parietal layer of the extraembryonic endoderm in conceptuses developing from blastocysts

Key words: immunosurgery, microsurgery, endoderm regeneration, primitive ectoderm, primitive endoderm, mouse blastocyst.

injected with one or more primitive endoderm cells (Gardner & Rossant, 1979; Gardner, 1982, 1984). The progeny of transplanted primitive ectoderm cells can be found in the extraembryonic mesoderm, the amniotic ectoderm and foetal tissues of the conceptus, and throughout the soma as well as in the germ line postnatally (Gardner, 1978*a*, 1982, 1983, 1984; Gardner & Rossant, 1979).

The precision with which the two types of donor cell segregate to different host tissues in the blastocyst injection experiments suggests that they have become restricted to mutually exclusive developmental pathways by 4·5 days *post coitum* (*p.c.*). However, the results of other experiments in which the technique of immunosurgery (IS) (Solter & Knowles, 1975) was used to destroy the endoderm of isolated ICMs in culture led Pedersen, Spindle & Wiley (1977) to conclude that primitive ectoderm retains the capacity to regenerate endoderm for a period of between 48 and 72 h after primitive endoderm differentiation has taken place.

Dziadek (1979) confirmed these *in vitro* findings and showed, in addition, that a much higher proportion of cells in the regenerated than the original endoderm reacted with an antiserum against α-foetoprotein, an established marker for visceral extraembryonic endoderm cells (Dziadek & Adamson, 1978). This led her to postulate that there were two phases of extraembryonic endoderm formation during normal development. The first phase was presumed to give rise to the parietal endoderm and to be followed somewhat later by the generation of the visceral endoderm from cells in the underlying primitive ectoderm (Dziadek, 1979).

Hence, the results of studies on isolated ICMs in culture are clearly at variance with those from the blastocyst injection experiments. However, several points should be taken into consideration in evaluating the *in vitro* experiments (Gardner, 1981). First, regeneration of endoderm has only been observed consistently in ICMs isolated from giant blastocysts produced by the aggregation of three to ten cleavage-stage embryos (Pedersen *et al.* 1977; Dziadek, 1979). Similar experiments on ICMs from standard blastocysts have yielded conflicting results. Thus, for example, while Hogan & Tilly (1977) found that immunosurgically-isolated ICM 'cores' invariably failed to regenerate endoderm, Atienza-Samols & Sherman (1979) observed that a small proportion could do so. Atienza-Samols & Sherman (1979) also noted that the proportion showing regeneration could be approximately doubled by pretreating the culture plates with medium conditioned by an endoderm-like cell line originating from a murine teratocarcinoma. They suggested that regeneration of endoderm by isolated ICM 'cores' *in vitro* required a critical mass of cells which could be achieved either by use of giant embryos or by medium conditioning.

Second, in all relevant experiments undertaken so far, embryos have been explanted into culture some time before cellular differentiation within the ICM seems to take place. Adverse effects of *in vitro* culture on both the growth and differentiation of ICM tissue have been reported (e.g. Bowman & McLaren, 1970; McReynolds & Hadek, 1972; Handyside, 1978; Spindle, 1978; Spielman, Jacob-Muller & Beckord, 1980). Therefore, it is conceivable that ICM 'core' cells

regenerating endoderm can do so because they have not attained the developmental status of primitive ectoderm cells.

Finally, the conclusion that ICM 'cores' were typically devoid of viable endoderm cells following immunosurgery was based entirely on inspection of intact or sectioned specimens by light microscopy (Pedersen *et al.* 1977; Atienza-Samols & Sherman, 1979; Dziadek, 1979). However, primitive endoderm cells can only be reliably identified ultrastructurally (Nadijcka & Hillman, 1974), or by light microscopy following dissociation (Gardner & Rossant, 1979). Therefore, the possibility that residual viable endoderm cells were responsible for regeneration cannot be discounted in these studies.

The initial aim of the present investigation was simply to find out if primitive ectodermal tissue recovered from *in vivo* at the stage when its cells appear by blastocyst injection to be restricted in developmental potential could regenerate an endodermal layer *in vitro*. The discovery that microsurgically (MS)-isolated primitive ectoderms typically failed to show regeneration while immunosurgically (IS)-treated ICMs did so routinely prompted a more detailed investigation of the effects of immunosurgery.

MATERIALS AND METHODS

Mice

Two stocks of mice were used which had been derived from the random-bred PO (Pathology, Oxford) albino strain. They differed in genotype at the glucosephosphate isomerase (*Gpi-1*) locus, one stock being $Gpi\text{-}1^a/Gpi\text{-}1^a$ and the other $Gpi\text{-}1^b/Gpi\text{-}1^b$. Use of embryos from the two stocks depended on availability in all *in vitro* experiments except those involving recombination of genetically dissimilar ectoderm and endoderm. The $Gpi\text{-}1^b/Gpi\text{-}1^b$ stock was invariably used to provide donor blastocysts and the $Gpi\text{-}1^a/Gpi\text{-}1^a$ stock both host blastocysts and pseudopregnant recipient females in the blastocyst injection experiments.

All mice were exposed to light between 07.00 and 19.00 h each day (= standard lighting regime) except part of the $Gpi\text{-}1^a/Gpi\text{-}1^a$ stock whose period of lighting extended from 23.00 to 14.00 h (= altered lighting regime). Oestrous females were selected (Champlin, Dorr & Gates, 1973) prior to onset of the dark period, caged overnight with fertile or vasectomized males of the same *Gpi-1* genotype, and checked for vaginal plugs the following morning. The day of the plug was recorded as the first day of pregnancy or pseudopregnancy. Pregnant females obtained using the altered light regime were assumed to be 6 h ahead of those on the standard one and were used as a source of late 4th and early 5th day blastocysts. In one series of experiments, exposure to males of oestrous females on standard lighting was delayed until the following morning so that mating could readily be timed to within 1 h (McLaren & Bowman, 1973). The oviducts and adjacent segments of uterus of pregnant females were flushed between 10.50 and 12.30 h on the third day to provide 4- to 8-cell embryos. Blastocysts were recovered at various times on the 4th and 5th day as stated in the Results. For the recovery of more advanced 5th day blastocysts each horn was inflated by clamping its oviductal end with forceps while introducing fluid via the cervix prior to repeated vigorous flushing. Even so, the recovery rate was typically about half that for the 4th day blastocysts. Finally, early postimplantation embryos were recovered by dissection with watchmaker's forceps of incipient decidual swellings from females killed between 10.50 and 15.45h on the 6th day of pregnancy.

Media

Embryos were recovered and manipulated in a modification of PBI medium (Whittingham & Wales, 1969) that has been described elsewhere (Gardner, 1982). Except where stated otherwise, culturing was done in microdrops under liquid paraffin (Boots Pure Drug Co., U.K.)

in bacteriological grade plastic dishes (Sterilin, U.K.) which were incubated at 37 °C in a humidified atmosphere of 5 % CO_2 in air. Aggregation of cleavage-stage embryos for the production of giant blastocysts was carried out in microdrops of standard ovum culture medium (Table 6.5 in Biggers, Whitten & Whittingham, 1971). Microdrops of α-medium (Stanners, Eliceiri & Green, 1971) supplemented with 10 % (v/v) foetal calf serum were used for the culture of ICMs and ICM derived tissue.

Microsurgery (MS)

All operations were done at a temperature of 11–12 °C using equipment that has been described elsewhere (Gardner, 1978*b*, 1984). For isolation of entire ICMs or primitive endoderm *versus* primitive ectoderm from 5th day blastocysts, the mural trophectoderm was first torn open with a pair of siliconized (Repelcote, Hopkin & Williams, U.K.) glass needles. The blastocysts were then incubated for 20 min at 4 °C in calcium-magnesium-free Tyrode's saline containing 0·5 % (w/v) trypsin and 2·5 % (w/v) pancreatin (both enzyme preparations from Difco, U.S.A.). This was followed by a 10–15 min incubation in 0·5 % pronase (w/v) (Calbiochem., U.S., grade B) in PBS, also at 4 °C. (In later experiments the incubation in trypsin/pancreatin was extended to 25 min while that in pronase was omitted.) Finally, after a brief rinse in chilled PBI, the blastocysts were returned to hanging drops in the manipulation chambers for further dissection.

The trophectoderm of the cold-enzyme-treated blastocysts was opened out fully with glass needles and its external surface flattened against the coverslip of the hanging drop. It was held thus to one side of the ICM region with one needle while the second was used gently to scrape the ICM away from the overlying polar trophectoderm. Isolation of the ICM was judged to be satisfactory if an intact layer of trophectoderm was left behind. The procedure for separating primitive endoderm from primitive ectoderm was essentially the same except the height of the second needle was adjusted so as to peel the endoderm away from the ectoderm. The ectoderm was then scraped away from the polar trophectoderm in a second step. The efficacy of separation of the primitive endoderm from the primitive ectoderm could not be monitored visually as clearly as that of the whole ICM or ectoderm from polar trophectoderm. It was regarded as satisfactory if the originally opposed surfaces of the separated tissues appeared smooth.

The procedure for transplanting tissue derived from the ICMs of 5th day blastocysts into the blastocoelic cavity of 4th day host blastocysts has been described fully elsewhere (Gardner, 1978*b*).

Immunosurgery (IS)

The procedure devised by Solter & Knowles (1975) was adopted with minor modifications (Nichols & Gardner, 1984) using antisera raised in rabbits against mouse spleen or foetal homogenate. When present, the zona pellucida was removed by exposing embryos briefly to acidified Tyrode's saline (Nicolson, Yanagimachi & Yanagimachi, 1975; Nichols & Gardner, 1984) before placing them in antiserum. Reichert's membrane was torn open and reflected prior to IS treatment of 6th day egg-cylinder-stage embryos, and the embryonic region of such embryos separated from the extraembryonic, either before or after their exposure to complement. The completeness of lysis of the outer cell layer in blastocysts or tissue fragments was checked routinely by inspecting treated specimens at a magnification of ×125 or ×312 while rotating them with a pair of blunt glass needles held in micromanipulators. The presence or absence of cellular debris had no discernible effect on the subsequent fate of IS-treated material in culture. Therefore, elimination of such debris was only attempted systematically in experiments in which specimens were to be aggregated or subjected immediately to a further round of IS. Direct transfer of tissue from complement to the trypsin–pancreatin solution described earlier for approximately 4 min at 4 °C prior to pipetting enabled rapid and complete removal of debris.

Aggregation of cleavage-stage embryos

Three denuded embryos containing between four and eight blastomeres were placed in each microdrop of pre-equilibrated standard ovum culture medium and incubated for approximately

1 h before being pushed together with a small glass probe. The embryos were inspected at intervals over the next 1–2 h to check the progress of aggregation and to re-establish contact if necessary. The aggregates were cultured for 48 h before those that had formed fully integrated giant blastocysts were exposed to IS or, in two cases, fixed for electron microscopy.

Cell counting and classification

MS-isolated primitive ectoderms and IS-treated ICMs were exposed to 0·5 % pronase for 15 min at 37 °C and then incubated in ovum culture medium lacking calcium until they had obviously decompacted (Nichols & Gardner, 1984). They were then dissected individually with blunt siliconized glass needles held in Leitz micromanipulators to enable both counting and morphological classification of the living cells.

Electron microscopy

Intact blastocysts and variously treated ICMs were fixed and embedded according to established procedures (Naeslund, Lundkvist & Nilsson, 1980). Sectioning was done on either a Huxley or LKB ultramicrotome, two ultrathin sections being taken after every 10th to 20th thick (1 μm) section, depending on the size of the specimen. The ultrathin sections were stained with uranyl acetate and lead citrate and examined in a Jeol JEM-100CX microscope. The 1 μm sections were stained with toluidine blue for examination by light microscopy.

Transplantation of blastocysts into pseudopregnant mice

Four to six 4th day blastocysts that had been injected with ICM tissue were transplanted into the oviductal end of one or both horns of females under Avertin (Winthrop, U.K.) anaesthesia in the late afternoon or early evening of the third day of pseudopregnancy.

Electrophoretic analysis

Uterine foster mothers were killed between the 11th and 13th day of gestation and their conceptuses dissected as described previously (Gardner & Rossant, 1979; Gardner, 1982). The structures formed *in vitro* following recombination of MS-isolated primitive ectoderms and primitive endoderms of opposite *Gpi-1* genotype were very variable in size and form. Nevertheless, all those that were integrated egg-cylinder- or cyst-like entities were cut in two and exposed to trypsin–pancreatin for approximately 15 min at 4 °C. Thereafter, they were returned to PBI and combined pipetting and dissection with watchmaker's forceps were used to separate the endoderm from the ectodermal derivatives.

Preparation, electrophoresis and staining of all samples were as described previously (Chapman, Whitten & Ruddle, 1971; Gardner, Papaioannou & Barton, 1973), except the smaller ones from cultured recombinant ICMs which were stained by the method of Peterson, Frair & Wong (1978) rather than with an agar overlay. The proportions of the two allozymes in chimaeric samples were assessed visually.

RESULTS

Endoderm regeneration in IS-treated ICMs from giant blastocysts

The ICMs were isolated by IS from giant blastocysts and then subjected to IS as shown in Table 1. Nearly all ICMs exhibited unequivocal regeneration of an external layer of endoderm, even if they had been subjected to IS immediately after isolation.

Isolation of ICMs from standard blastocysts recovered at different stages

In preliminary experiments IS was used to obtain whole ICMs from blastocysts of all stages but in the case of embryos recovered from the uterus around noon or

later on the 5th day of pregnancy this technique was soon abandoned for two reasons. First, the lysed trophectoderm could not be eliminated simply by pipetting (Solter & Knowles, 1975) in these more mature blastocysts. Hence, ICMs had to be freed from trophectoderm debris by MS. Second, even when divested of debris satisfactorily prior to further IS treatment, such ICMs were almost invariably found to be partially surrounded by a tough transparent membrane thereafter. This membrane, which may be the precursor of Reichert's membrane, often appeared to invest a layer of viable endoderm cells, suggesting that its presence may have impaired lysis of these cells. ICMs isolated by MS were devoid of such a membrane, presumably as a result of exposure to trypsin-pancreatin solution. Therefore, MS was used to isolate ICMs from the more advanced 5th day blastocysts.

Endoderm regeneration in IS-treated ICMs versus *MS-isolated primitive ectoderms from standard blastocysts*

MS-isolated primitive ectoderms and IS-treated ICMs from 5th day blastocysts were scored, at intervals during culture for up to one week, for formation of an outer 'rind' of endoderm. The frequency with which the two types of tissue fragments formed such a layer is indicated in Table 2, together with the corresponding frequencies for intact ICMs and ICMs produced by recombining primitive endoderms with primitive ectoderms shortly after their separation by MS. At least 57 % of IS-treated ICMs regenerated an endoderm layer (Table 2, rows 7 & 8) whereas only 4 % of MS-isolated primitive ectoderms did so (Table 2, rows 1–3). The rate of regeneration shown by the two types of tissue fragment was significantly different regardless of whether they were cultured singly or in aggregates of two or three (Table 2, see footnotes). Primitive ectodermal tissue usually degenerated completely before the end of the culture period (Fig. 1) Failure of this tissue to survive was clearly not due to the culture conditions *per se* since it often grew vigorously in intact and IS-treated ICMs (Figs 2 & 3). Nor was degeneration attributable to damage during MS because growth of ectoderm following its successful recombination with endoderm rivalled that in intact ICMs (Figs 3 & 4). The marked difference in survival between IS-treated ICMs and

Table 1. *Regeneration of endoderm following IS treatment of ICMs from giant blastocysts*

Treatment of ICMs*	No. of ICMs	No. (%) ICMs regenerating endoderm
IS after culture for 37 h following isolation	4	4(100)
IS after culture for 20 h following isolation	17	15(88)
IS immediately after isolation	9	8(89)

* ICMs were isolated by IS from integrated giant blastocysts that had formed approximately 48 h after the onset of aggregation of 4- to 8-cell embryos in threes.

Table 2. *Formation of endoderm by cultured MS-isolated primitive ectoderms* versus *IS-treated ICMs from blastocysts recovered on the fifth day of pregnancy*

	Tissue cultured	Total of specimens cultured	No. forming unequivocal endoderm layer
†(1)	MS-isolated primitive ectoderms* cultured singly	36	2
††(2)	MS-isolated primitive ectoderms* aggregated in pairs	10	0
††(3)	MS-isolated primitive ectoderms* aggregated in threes	7	1/3×1***
(4)	Recombined single MS-isolated primitive ectoderms and primitive endoderms	16	15
(5)	MS-isolated whole ICMs	29	29
(6)	IS-isolated whole ICMs	14	14
†(7)	MS- or IS-isolated whole ICMs exposed to IS and cultured singly**	72	52
††(8)	MS-isolated whole ICMs exposed to IS and aggregated (7×2 plus 1×3)	8	8****

† One significantly different from seven $P < 0{\cdot}001$.

†† Two plus three significantly different from eight $P < 0{\cdot}001$.

* Seventy-one of the corresponding MS-isolated primitive endoderms were cultured, three of which were later observed to contain definite 'cores' of presumed primitive ectoderm. A further six specimens were classified as possibly containing such 'cores'.

** IS was used to isolate ICMs from 28 blastocysts recovered between 06.50 and 07.20h on the fifth day. Twenty-six of the ICMs from these blastocysts regenerated endoderm following their exposure to IS. MS was used to isolate ICMs from the remaining 44 blastocysts which were recovered later on the 5th day.

*** Approximately two thirds of this spherical mass of cells degenerated while the remainder persisted as a coherent cluster enveloped by endoderm. Hence, only one of the three ICMs comprising the aggregate was presumed to have regenerated endoderm.

**** This is a minimum figure because only one member of each aggregate was asssumed to have formed the entire new layer.

isolated primitive ectoderms also cannot be accounted for by disparity in cell mass. The number of cells in MS-isolated ectoderms (mean S.D. = 23·1 ± 7·2; N = 17) was found to be similar to that in IS-treated ICMs (mean S.D. = 21·4 ± 8·6; N = 20) and an approximate doubling or trebling of the number by aggregating ectoderms only delayed rather than prevented their degeneration.

Endoderm differentiated into a prominent external layer in all intact ICMs and in all but one of the recombinant ICMs (Table 2, rows 4–6, and Figs 3 & 4). In addition all 71 MS-isolated primitive endoderms that were cultured survived throughout incubation, many of them increasing substantially in size (Fig. 5). Ectoderm-like 'cores' were definitely present in three cultured endoderms and possibly present in an additional six specimens (Table 2, footnote).

Marked differences in aggregative capacity of the various categories of 5th day ICM fragments were noted during these culture experiments. MS-isolated primitive endoderms showed no tendency to aggregate with each other despite the

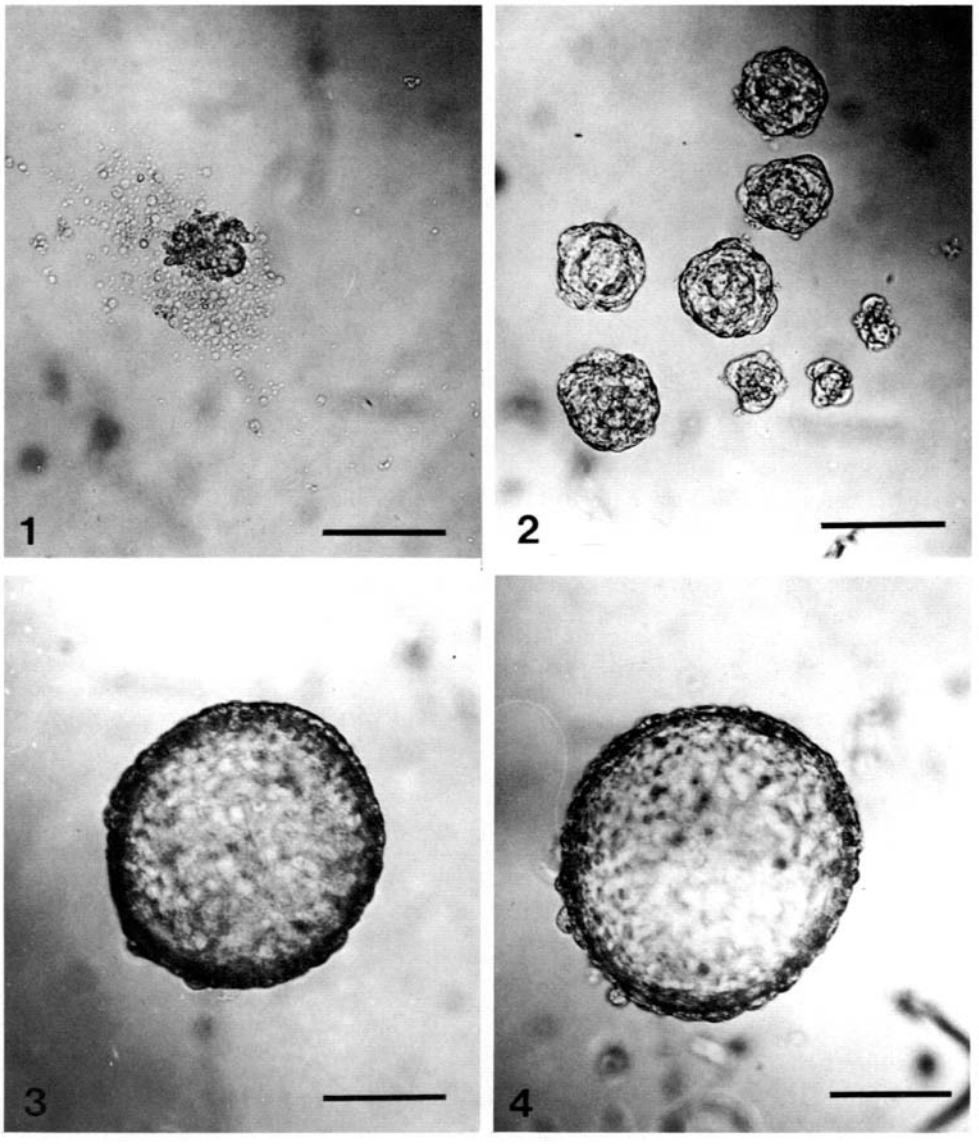

Fig. 1. Aggregated pair of MS-isolated 5th day primitive ectoderms after 2 days in culture. Note the extensive cellular debris and the lack of an external layer of endoderm in the residual mass of cells that have not yet degenerated.

Fig. 2. A group of IS-treated 5th day ICMs after 2 days in culture. Cellular debris is absent and an external layer of endoderm is discernible in the majority of cases.

Fig. 3. An expanded bilaminar vesicle formed by an intact isolated 5th day ICM photographed on the 3rd day of culture.

Fig. 4. A recombinant ICM on the 3rd day of culture following reaggregation of ectoderm and endoderm isolated by MS from a 5th day blastocyst showing a similar bilaminar vesicular structure to the intact ICM illustrated in Fig. 3. Bars in Figs 1–4 = 50 μm.

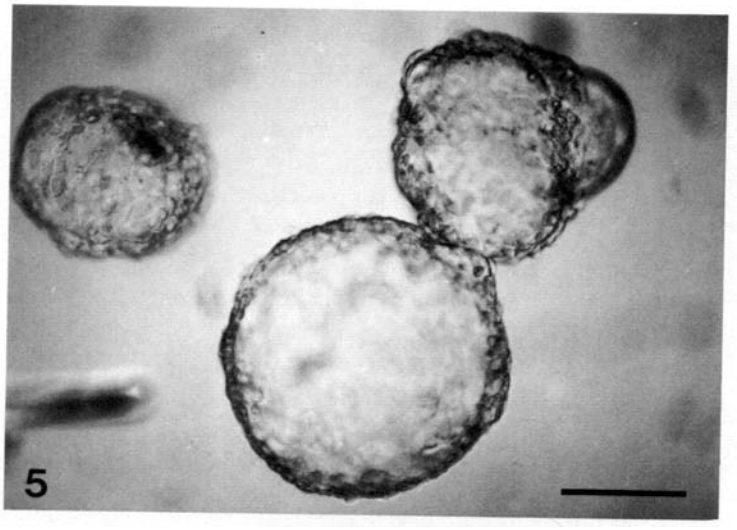

Fig. 5. Three unilaminar vesicles formed by MS-isolated endoderms from 5th day blastocysts that had been cultured for 5 days. Bar = 50 μm.

facility with which they enveloped primitive ectoderms to form recombinant ICMs (Table 2, row 4, and Fig. 5). Pairs or groups of MS-isolated primitive ectoderms typically aggregated rapidly to form unitary spherical or nearly spherical cell masses within 3–5 h of being placed in contact. IS-treated ICMs, in contrast, resembled intact ones in aggregating much less readily, if at all, so that the boundaries of participating fragments were often discernible throughout culture.

The effect on endoderm regeneration of varying the stage of recovery and IS treatment of ICMs was examined in three additional series of experiments (Table 3A, B & C). A significant reduction in the frequency of regeneration using two as opposed to one round of IS on newly isolated ICMs was only observed with 5th day specimens (footnote to Table 3C).

IS-treated ICMs were found to differ from MS-isolated primitive ectoderms in cellular composition as well as in capacity for endoderm regeneration and aggregation. Each of 20 IS-treated ICMs were found following decompaction to contain between three and seventeen cells exhibiting the 'rough' appearance characteristic of primitive endoderm (Gardner & Rossant, 1979), whereas such cells were encountered in only 2 out of 17 MS-isolated ectoderms (1 ectoderm contained one and the other two R cells). The failure of IS to destroy all endoderm cells in treated ICMs did not seem to be attributable to deficiencies in either the reagents or procedure. All ICMs that were inspected closely immediately after IS appeared to have a completely lysed external layer of cells. In addition, IS yielded very similar results to MS with regard to regeneration of endoderm in early postimplantation embryos (Table 4). Hence it seemed likely that some endoderm cells were being shielded from destruction by IS in 5th day ICMs. It was necessary to resort to electron microscopy to investigate this possibility.

Table 3. *Investigation of the effect of varying stage and IS treatment of ICM tissue on the frequency of endoderm regeneration*

(A) ICMs isolated by IS from 4th day blastocysts and aggregated in twos or threes for 10·5–25 h in culture prior to a further round of IS (controls exposed to antiserum alone).

Time of recovery of blastocysts on 4th day	No. of ICMs per aggregate	Duration of culture of aggregates prior to IS	No. of aggregates regenerating endoderm/ total cultured	No. of control aggregates regenerating endoderm/total cultured
ca. 14.00 h	3	25 h	5/5	—
ca. 17.30 h	2	24 h	9/10	3/4
ca. 16.00 h	3	14·5 h	5/5	4/4
ca. 17.30 h	2	10·5–12·5 h	12/13	—

(B) ICMs isolated by IS from 4 day* or earlier blastocysts and cultured in pairs for the minimum period needed to obtain aggregation (1 h 50 min–2 h 50 min) prior to two further rounds of IS (controls exposed to one full additional round of IS plus to antiserum only in the second round).

Time of recovery of blastocysts	No. of aggregates regenerating endoderm/total no. of experimental aggregations	No. of aggregates regenerating endoderm/total no. of control aggregates
14.15–16.00 h on 4th day	26/40	8/10
4 days p.c.	14/35	4/4

* These blastocysts were recovered 96 h after timed delayed mating

(C) ICMs isolated by IS or MS and subjected to two immediate rounds of IS (controls exposed to one full additional round of IS plus antiserum only in the second round).

Time of recovery of blastocysts	No. of ICMs regenerating endoderm/total no. of experimental ICMs	No. of ICMs regenerating endoderm/total no. of control ICMs
ca. 22.00 h on 4th day	7/14	10/10
ca. 15.00 h on 5th day	4/17**	9/13

** $0{\cdot}05 < P < 0{\cdot}02$ compared to controls

EM observations

The number and treatment prior to fixation of the various types of specimens examined are recorded in Table 5, together with comments on their status with regard to relatively RER-rich presumed primitive endoderm cells. The principal findings may be summarized as follows. The endoderm was clearly more than one cell thick in the intact 5th day blastocysts, particularly towards the lateral edge of the ICM adjacent to the mural trophectoderm, where it could be as much as three to four cells deep (Fig. 6). Furthermore, it appeared to be arranged asymmetrically, being thicker one side of the ICM than the other in most sections. Conspicuous multilayering of endoderm was also apparent in isolated 5th day ICMs that had been cultured until they formed a complete external rind of these

cells (Table 5, row 3, and Fig. 7). It was perhaps not surprising, therefore, that intact endoderm cells were seen between the ectodermal core and external layer of lysed cells in the majority of cultured 5th day ICMs that were fixed immediately after IS (Table 5, row 4 and Fig. 8). No candidates for endoderm cells were seen in MS-isolated primitive ectoderms (Table 4, row 2).

The two giant blastocysts examined did not contain any ICM cells that were as well endowed with RER as standard 5th day blastocysts. Nevertheless, relatively

Table 4. *Investigation of endoderm regeneration by 6th day MS* versus *6th day IS-isolated embryonic ectoderm*

Tissue fragments		Fate in culture		
Type	No. of specimens cultured	No. degenerating	No. partially surviving	No. forming bilaminar structures
MS-isolated ectoderm	5	5	0	0
IS-isolated ectoderm*	26	24	2†	0
Ectoderm invested with endoderm	9	0	0	9

* Three additional IS-isolated ectoderms that were cultured in mitomycin C-treated STO feeder cells grew vigorously, one developing rhythmic contractile activity.
† One of these persisted as a thin-walled vesicle that superficially resembled a giant blatocyst, and the other as a much smaller vesicle containing a pycnotic core.

Table 5. *Specimens examined in the electron microscope*

Type of specimen	Treatment	No. examined	Comments
(1) Intact 5th day blastocyst	Fixed on recovery	3	All showed multi-layering of RER-rich cells
(2) MS-isolated primitive ectoderms from 5th day blastocysts	Fixed following isolation	6	All lacked unequivocal RER-rich cells
(3) MS-isolated whole ICMs from 5th day blastocysts	Cultured until they had formed a conspicuous rind of endoderm (ca. 24 h) prior to fixation	3	All showed multi-layering of RER-rich cells
(4) MS-isolated whole ICMs from 5th day blastocysts	Cultured for 15 h and then fixed immediately after IS	5	4 had viable-looking RER-rich cells inside lytic halo
(5) Intact giant blastocysts	Fixed ca. 48 h after aggregation of 3×4- to 8-cell embryos	2	ICMs of both contained RER-rich cells which seemed to be multi-layered in one
(6) IS isolated whole giant ICMs	Cultured for 37 h and then fixed immediately after IS	2	Both had viable-looking RER-rich cells inside lytic halo

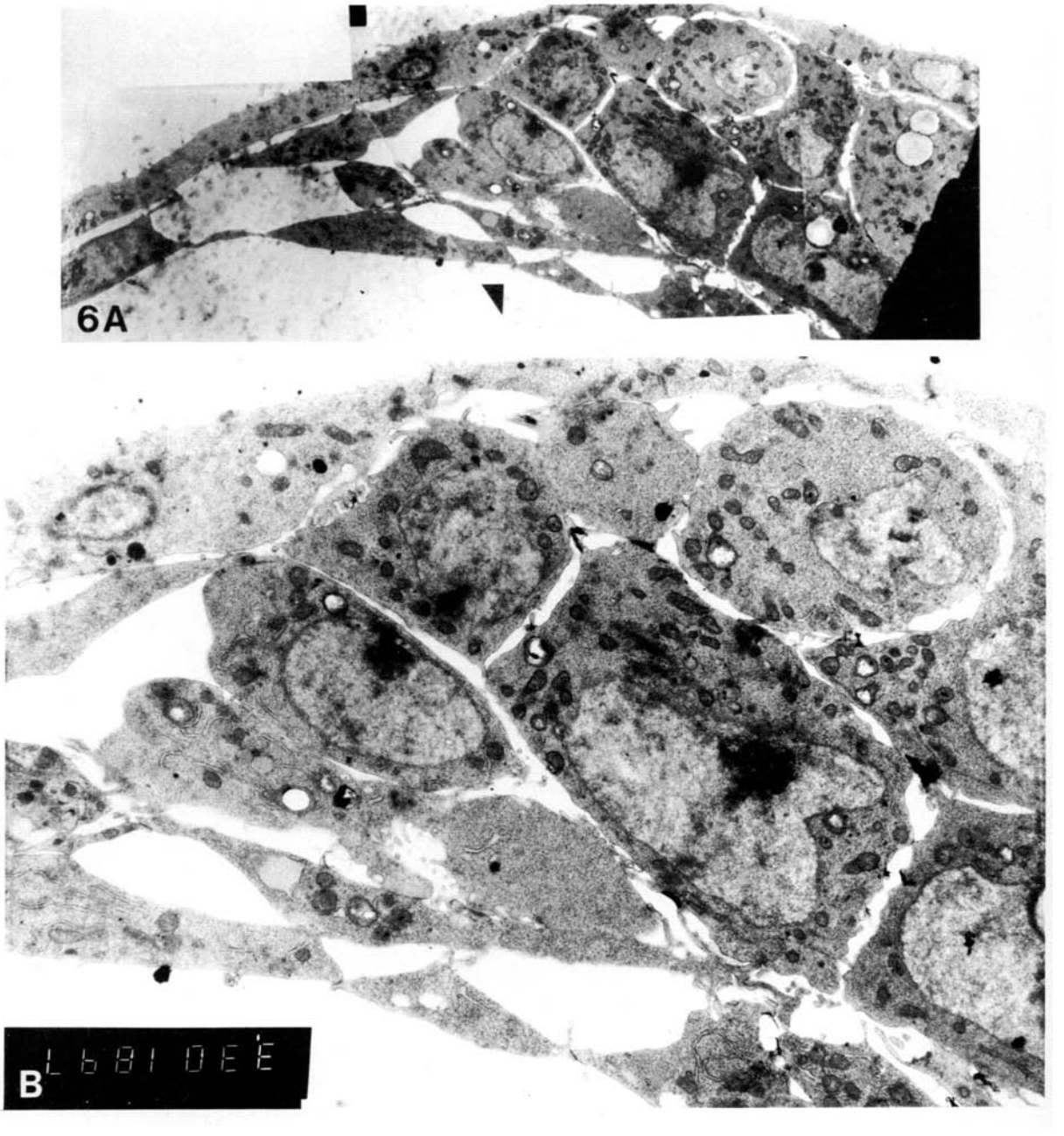

Fig. 6. (A) Part of the ICM region of an intact 5th day blastocyst sectioned parallel to the embryonic–abembryonic axis (×3600) (B) Detail of A at higher magnification to show multilayering of RER-rich presumed primitive endoderm cells (×7660).

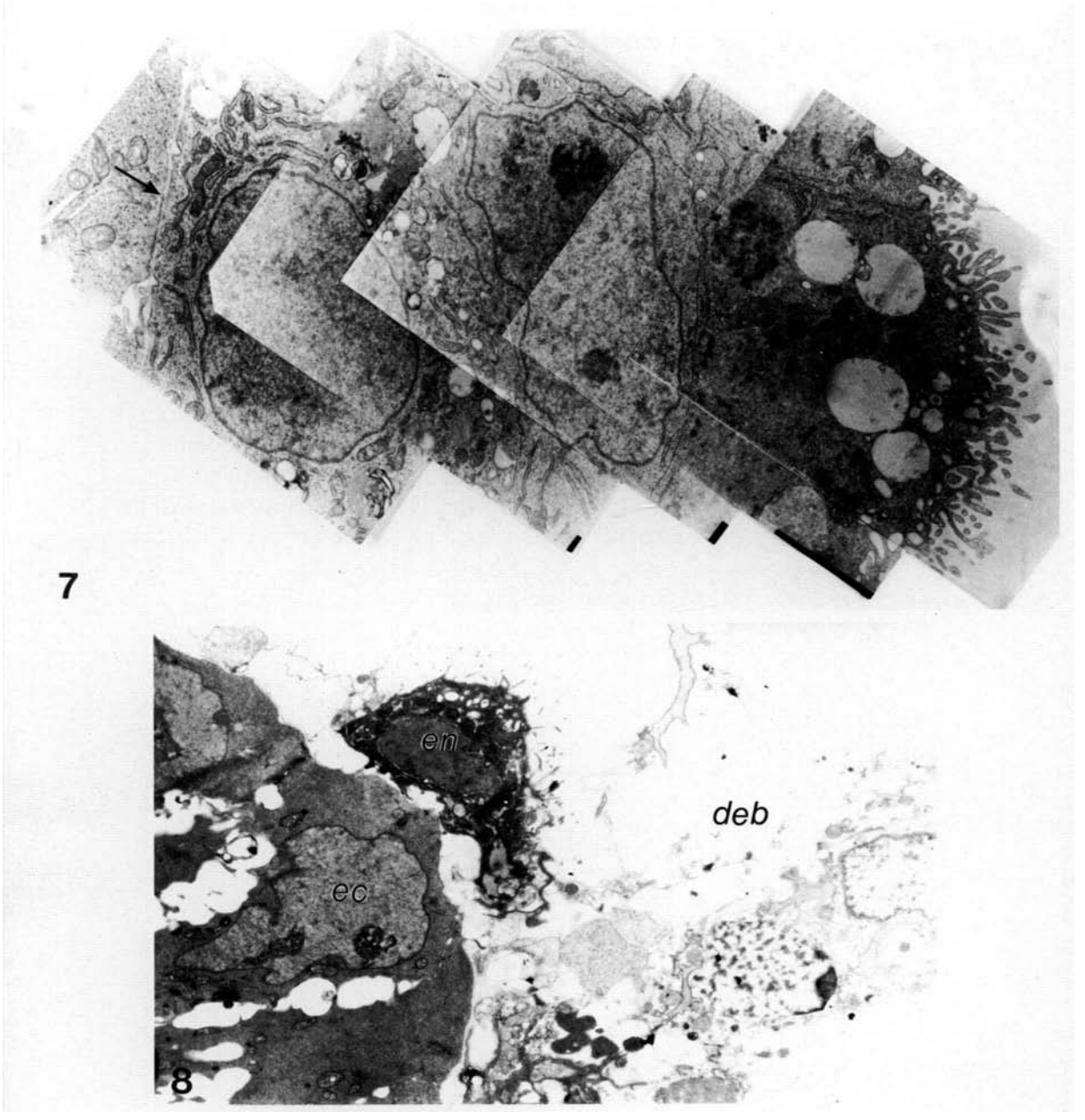

Fig. 7. Montage of part of the endoderm of an intact 5th day ICM that was cultured for approximately 24 h so that it formed a conspicuous external layer of endoderm prior to fixation. The endoderm is three cells thick in this region with the basal cell adjacent to the ectoderm being particularly well-endowed with RER. Arrow indicates the junction between endoderm and ectoderm (×6320).

Fig. 8. Part of a recently isolated 5th day ICM that was fixed immediately after IS. An intact presumed endoderm cell (*en*) lies between the ectoderm (*ec*) and the debris (*deb*) of lysed endoderm cells (×4300).

RER-rich cells appeared to be present in more than just the superficial layer in one. Candidates for viable endoderm cells were also seen in both cultured giant ICMs fixed immediately after IS (Table 5, row 6).

Origin of regenerated endoderm in recombinant ICMs

The experimental protocol is outlined in Fig. 9. A total of 34 recombinant ICMs consisting of MS-isolated primitive ectoderms and endoderms of different *Gpi-1* genotype were established in culture. Two failed to grow and were therefore discarded prior to IS. A further two degenerated following IS without regenerating an endoderm layer. Three of the remaining 30 grew into amorphous masses in which no differentiation could be discerned. All the rest of the recombinants clearly regenerated an endoderm layer, but three of them failed to attain a dissectable size. No enzyme activity was obtained from either the endodermal or ectodermal component of four of the 24 that were dissected. Unequivocal GPI typing was achieved for one or both tissues from the remaining 20 recombinants, as shown in Table 6. The regenerated endoderm appeared to have been derived entirely from the original endoderm in 17 of the 19 recombinants in which GPI activity was detected in this fraction. The remaining two regenerated endoderms

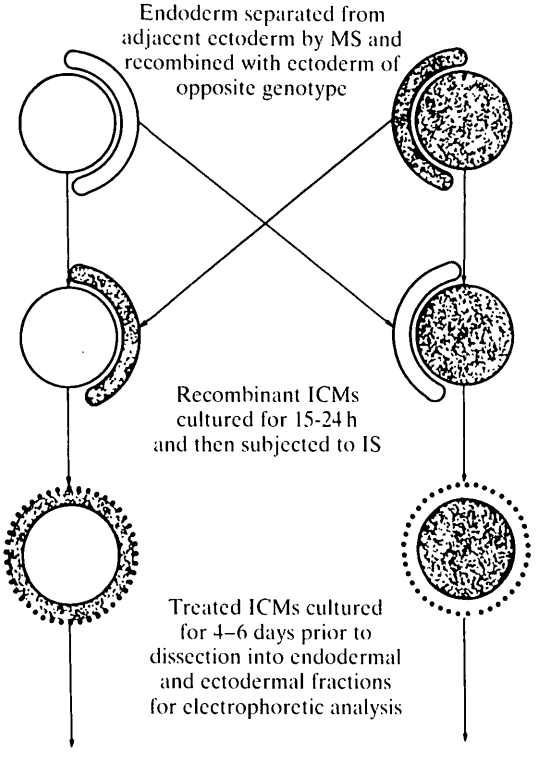

Fig. 9. Protocol of recombinant ICM experiment.

were clearly composed of cells derived from both the original endoderm and ectoderm, as was one of the corresponding ectodermal fractions (Table 6, Nos. 6 & 19). The genotype of the ectodermal fractions was as expected in the remaining eight cases in which they could be typed.

Developmental potential of 5th day ICM tissue in vivo

Table 7A gives the incidence and distribution of chimaerism in conceptuses obtained from blastocysts that had been injected with MS-isolated primitive ectoderms, MS-isolated primitive endoderms, or IS-treated ICMs. The MS-isolated ectoderms and endoderms colonized only primitive ectodermal and primitive endodermal derivatives, respectively, in the great majority of cases. IS-treated ICMs contributed cells to primitive endodermal derivatives in host conceptuses significantly more frequently than did MS-isolated ectoderms (see footnote, Table 7A) and, indeed, typically behaved like a combination of primitive ectodermal and primitive endodermal tissue.

The distribution of donor cells within the extraembryonic endoderm is shown for all conceptuses in which this component was chimaeric in Table 7B. IS-treated ICMs closely resemble MS-isolated endoderms in incidence of colonization of the parietal and visceral cell layers.

Table 6. *Origin of regenerated endoderm in IS-treated recombinant ICMs*

Number of recombinant	GPI type of original		GPI type of regenerated	
	endoderm	ectoderm	endoderm	ectoderm
1	B	A	B	A
2	B	A	B	na
3	B	A	B	na
4	B	A	B	na
5	B	A	B	na
6	B	A	A+B(1/1)	A+B(3/1)
7	B	A	na	A
8	B	A	B	na
9	B	A	B	na
10	A	B	A	na
11	A	B	A	B
12	A	B	A	na
13	A	B	A	B
14	A	B	A	na
15	A	B	A	na
16	A	B	A	B
17	A	B	A	?B*
18	A	B	A	B
19	A	B	A+B(12/1)	B
20	A	B	A	B

na = no GPI activity recorded.
* too little activity to score confidently.

Table 7. *Distribution of chimaerism in conceptuses developing from 4th day blastocysts injected with ICM tissue from 5th day blastocysts*

(A) Overall chimaerism

Type of donor tissue	Blastocysts		Normal conceptuses		Tissues colonized by donor cells*		
	Total no. transplanted	No. transplanted to recipients that became pregnant	Total no.	No. chimaeric	Primitive ectoderm derivatives only	Primitive endoderm derivatives only	Primitive ectoderm and primitive endoderm derivatives
MS-isolated*** primitive ectoderm	40	35	24	21	19	0	2
MS-isolated*** primitive endoderm	37(8)**	26(8)**	18(7)**	18(7)**	0	17(6)**	1(1)**
IS-treated*** entire ICM	51	45	33	33	4	4	25

(B) Chimaerism in extraembryonic endoderm

Type of donor tissue	No. of conceptuses exhibiting extraembryonic endodermal chimaerism	Distribution of donor cells in extraembryonic endoderm		
		Parietal endoderm only	Visceral endoderm only	Parietal and visceral endoderm
MS-isolated primitive endoderm	2	0	1	1
MS-isolated primitive endoderm	18(7)**	2(1)**	0	16(6)**
IS-treated entire ICM	29	4	0	25

* Primitive ectoderm derivatives assayed were foetus, amnion, umbilical cord, and visceral yolk sac mesoderm. Primitive endoderm derivatives assayed were parietal endoderm and visceral yolk sac endoderm (see Gardner, 1983, for details of these lineages).

** Numbers in brackets in both part A and B of the table relate to an experiment in which half an MS isolated endoderm was injected into each blastocyst.

*** Difference in frequency of colonization of extraembryonic endoderm by MS-isolated ectoderm (2/21) *versus* IS-treated ICMs (29/33) significant. $P < 0{\cdot}001$.
Difference in frequency of colonization of extraembryonic endoderm by MS-isolated endoderms (18/18) *versus* IS-treated ICMs (29/33) not significant.

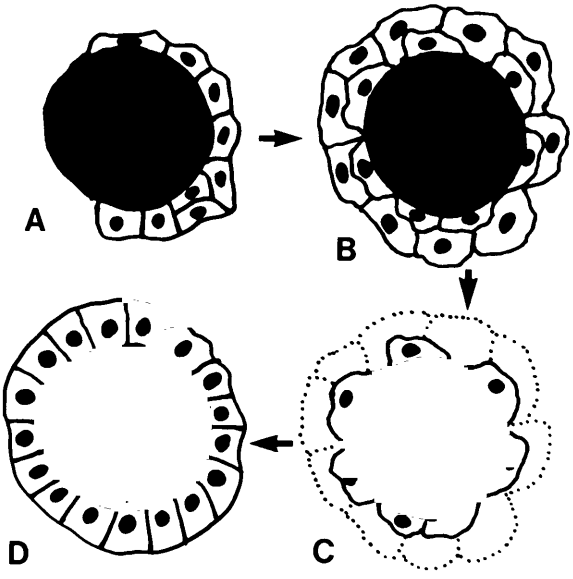

Fig. 10. Scheme to account for regeneration of an external layer of endoderm following IS-treatment of mature ICMs. (A) Recently isolated ICM with endoderm cells confined to its original blastocoelic surface. (B) Culture of such an ICM for the 24 h or so required for it to form a complete external 'rind' of endoderm is accompanied by more extensive multilayering of cells within this tissue. (C) Hence, when such cultured ICMs are exposed to IS some endoderm cells are physically protected by others from exposure to antiserum. (D) Consequently, a new external layer of endoderm can be formed from the residual viable endoderm cells.

DISCUSSION

The present results confirm those of certain earlier studies (Pedersen *et al.* 1977; Atienza-Samols & Sherman, 1979; Dziadek, 1979) in showing that generation of a new external layer of endoderm can take place in isolated ICMs in culture following destruction of the original layer by IS. They differ in demonstrating a much higher rate of reformation of a layer in ICMs from standard blastocysts than reported hitherto, a difference that is probably attributable to the fact that donor embryos were allowed to complete their maturation *in vivo* in the present investigation.

If IS is a reliable method of destroying the entire endoderm, as was assumed rather than proven in previous studies, treated ICMs might be expected to resemble MS-isolated primitive ectoderms in developmental potential. This is clearly not the case. MS-isolated ectoderms exhibited a significantly lower rate of endoderm regeneration *in vitro* and colonization of extraembryonic endoderm *in vivo* than IS-treated ICMs (Tables 2 and 7). The marked disparity in behaviour of the two types of tissue fragments *in vitro* cannot readily be ascribed to the lack of a critical mass of viable cells in the MS-isolated ectoderms (Pedersen *et al.* 1977; Atienza-Samols & Sherman, 1979). The ectoderms appeared to rival IS-treated

ICMs in cell number and their regenerative potential was not enhanced if they were aggregated prior to culture. Furthermore, since such ectoderms continued to grow if recombined with endoderm or injected into blastocysts following isolation, their consistent degeneration *in vitro* cannot be attributed to damage during microsurgery. It is noteworthy that primitive ectoderm from postimplantation embryos is also unable to survive in simple culture conditions despite its greater cell mass, unless invested by endoderm or given a suitable feeder cell layer (see Table 4, and Rossant & Ofer, 1977).

MS-isolated ectoderms clearly yielded endoderm in a small minority of cases in both *in vitro* and *in vivo* experiments. However, since the frequency with which they did so was very similar to that with which corresponding endoderms produced ectodermal tissue (see Tables 2 and 7), the phenomenon is more readily ascribed to occasional failure to separate the tissues cleanly than to alteration in cell fate.

The greater difficulty experienced in aggregating IS-treated ICMs than MS-isolated ectoderms suggested that the two types of tissue fragment might differ in cellular composition. This was confirmed by detection of relatively RER-rich cells in the majority of EM preparations of IS-treated ICMs and identification of cells of characteristic 'rough' appearance (Gardner & Rossant, 1979; Gardner, 1982, 1984) in each of a series of dissociated living specimens. Such morphological indices of the presence of endoderm were evident only exceptionally in corresponding preparations of MS-isolated ectoderms.

Ineffectiveness of the reagents or inaccessibility of some cells to antiserum are two obvious explanations for the persistence of apparently viable endoderm in IS-treated ICMs. Absence of discontinuities in the halo of lysed cells in both living specimens and EM preparations of treated ICMs made it unlikely that external cells had escaped destruction routinely. The possibility that some endoderm cells were indeed being shielded from exposure to antiserum gained credibility with the discovery that the endoderm is typically more than one cell layer thick in mature ICMs. Multilayering of cells in the endoderm is even more conspicuous if, as in the experiments of Pedersen *et al.* (1977) and Dziadek (1979), isolated mature ICMs are cultured intact for 24 h prior to IS (Table 5 and Fig. 7). IS was clearly as effective as MS for isolating ectoderm from 6th day egg cylinders (Table 4) whose endoderm is definitely single layered (Reinius, 1965; Solter, Damjanov & Skreb, 1970; Bartel, 1972). This finding also supports the conclusion that inaccessibility of cells rather than inadequacy of reagents was responsible for the failure of IS to eliminate the entire endoderm from 5th day ICMs.

Pedersen *et al.* (1977) also reported that endoderm cells could survive IS in a proportion of ICMs from giant blastocysts. However, they argued on the basis of the rate of decline in nuclear grain counts following brief labelling of IS-treated giant ICMs with [^{3}H]thymidine, that proliferation of contaminating endoderm cells was insufficient to account for the regeneration of an external endoderm layer. The question whether residual endoderm could be responsible for regeneration was examined more directly in the present study, by typing the endoderm layer obtained following IS-treatment of ICMs composed of genetically

dissimilar endoderm and ectoderm. In all except two of the nineteen recombinant ICMs in which the regenerated endoderm could be typed, it was found to consist entirely of cells from the original endoderm. One of the two exceptions (Table 6, No. 6) was probably attributable to failure to separate the regenerated endoderm clearly from the underlying ectoderm prior to assay, since both fractions were conspicuously chimaeric. Only the endoderm fraction was chimaeric in the other, and contained a small minority of ectoderm-derived cells (Table 6, No. 19). The possibility that this is a genuine case of cellular respecification obviously cannot be excluded, although, as discussed earlier, it is more likely to be the result of endoderm contamination of the ectodermal component in the original recombinant ICM.

The present findings are consistent with the following explanation for the striking disparity in the results obtained using IS *versus* MS to isolate the primitive ectoderm from mature ICMs. MS normally yields ectoderms that are devoid of contaminating endoderm cells because trypsin–pancreatin treatment of ruptured blastocysts seems to weaken selectively the union between the two tissues. Such ectoderms show a similar dependence on the presence of other types of cell for survival as their postimplantation derivatives (Rossant & Ofer, 1977). IS, in contrast, is presumed to be effective in destroying external cells only, and will therefore not produce pure ectoderms unless, as in the case of 6th day egg cylinders, the endoderm is single layered throughout. Since the endoderm appears normally to be at least two layers thick in ICMs from 5th day blastocysts that have developed *in vivo*, a single round of IS seems to leave sufficient endoderm cells intact to ensure both survival of the ectoderm and generation of a new external epithelium (Fig. 10). Indeed, even two successive rounds of IS failed to abolish regeneration in all cases, though it did result in a significant decrease in its frequency of occurrence (Table 3). As noted earlier, past failure to obtain regeneration of endoderm consistently in IS-treated ICMs (Hogan & Tilly, 1977; Pedersen *et al.* 1977; Atienza-Samols & Sherman, 1979) has been attributed to lack of a critical mass of cells in the remaining ectoderm (Pedersen *et al.* 1977; Atienza-Samols & Sherman, 1979). However, according to the explanation for regeneration presented here, it is the extent of survival and subsequent growth of endoderm that is decisive. If this is indeed the case, the beneficial effects of precoating culture plates with medium conditioned by an endoderm-like cell line (Atienza-Samols & Sherman, 1979) is presumably exerted primarily via an effect on supposedly homotypic rather than heterotypic cells.

The finding that cells were more often AFP-positive in the regenerated than the original endoderm layer in giant ICMs prompted Dziadek (1979) to equate primitive with parietal endoderm and to propose a separate origin from primitive ectoderm for visceral endoderm. The conclusion of Pedersen *et al.* (1977) that primitive ectoderm cells do not become determined until between 48 and 72 h after their initial appearance was based on investigating the length of time for which giant ICMs could be cultured prior to IS and still regenerate an endoderm layer thereafter. The present findings obviously challenge interpretation of the data in

the foregoing studies by casting serious doubt on the validity of the assumption that primitive ectoderm gave rise to the regenerated endoderm. There are additional reasons for questioning the main conclusions drawn from both the above studies concerning cell lineage and determination in the ICM.

The principal objections to the lineage scheme proposed by Dziadek (1979) are as follows:

1. Blastocyst injection experiments have provided no evidence for the existence of cells in mature ICMs that can colonize primitive ectoderm derivatives plus visceral endoderm, specifically. Such experiments have, however, demonstrated unequivocally that individual primitive endodermal cell clones can embrace both the parietal and visceral endoderm (Gardner, 1982, 1984).

2. As shown in Table 7B, MS-isolated endoderms and IS-treated ICMs did not show preferential colonization of parietal and visceral endoderm, respectively, following injection into host blastocysts. Instead, both types of fragment exhibited more or less equal contributions to the two extraembryonic endoderm layers in the great majority of cases.

3. A positive correlation in mosaicism between parietal and visceral endoderm has been reported in a series of aggregation chimaeras at midgestation. No such correlation was seen between either of these tissues and those of primitive ectodermal origin (West, Bucher, Linke & Dunnwald, 1985).

Therefore, there are compelling grounds for rejecting Dziadek's lineage scheme as untenable. The above data are, however, clearly consistent with an earlier proposal that parietal and visceral endoderm share a common origin from the primitive endoderm (Gardner & Papaioannou, 1975).

The fact that individual 5th day primitive ectoderm cells can give rise to both somatic cells and functional gametes (Gardner, 1978*a*) argues that they retain genomic totipotency. Nevertheless, results obtained in the present study and in earlier blastocyst injection experiments (Gardner & Rossant, 1979; Gardner, 1982) do not support the conclusion that these cells remain undetermined until 2 days or so after implantation. Rather, they suggest that both primitive ectoderm more or less synchronously, some time between $3\frac{3}{4}$ and $4\frac{1}{2}$ days *post coitum*. So far, extraembryonic-endoderm-like cells have only been obtained unequivocally from primitive ectoderm following its exposure to conditions of teratocarcinogenesis (Graham, 1977). In view of current ignorance of events underlying this process and uncertainty as to whether the target for neoplastic conversion is the primitive ectoderm cell itself or one of its lineal descendants, it is not clear what relevance, if any, such findings have to the problem of stability of ectodermal determination. There are also indications that primitive-endoderm-derived cells may exhibit metaplasia in certain circumstances (Lu, Sobis, Van Hove & Vandeputte, 1984). and primitive endoderm cells become restricted to mutually exclusive lineages Nevertheless, it would appear that both types of mature ICM cell have undergone sufficiently stable differentiation by the late blastocyst stage to render their interconversion during subsequent embryogenesis a most unlikely event.

Finally, two questions that remain unanswered in the present study are why endoderm is more conspicuously AFP-positive in IS-treated than untreated ICMs *in vitro* (Dziadek, 1979), and why regeneration fails if it is postponed beyond a certain interval (Pedersen *et al.* 1977; Dziadek, 1979). The answer to the first question will depend on a better understanding of factors determining the onset of synthesis of AFP during embryogenesis. Regarding the second, eventual exteriorization of all endoderm cells or loss of proliferative capacity of those that remain trapped internally, are among the more obvious explanations.

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REFERENCES

ATIENZA-SAMOLS, S. B. & SHERMAN, M. I. (1979). *In vitro* development of core cells of the inner cell mass of the mouse blastocyst: effects of conditioned medium. *J. exp. Zool.* **208**, 67–71.

BARTEL, H. (1972). Electron microscopic observations of the inner cell mass of a mouse embryo. *Acta Anat.* **83**, 289–301.

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

BOWMAN, P. & MCLAREN, A. (1970). Cleavage rate of mouse embryos *in vivo* and *in vitro*. *J. Embryol. exp. Morph.* **24**, 203–207.

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.

CHAPMAN, V. M., WHITTEN, W. K. & RUDDLE, F. H. (1971). Expression of paternal glucose phosphate isomerase-1 (Gpi-1) in preimplantation stages of mouse embryos. *Devl Biol.* **26**, 153–158.

DZIADEK, M. (1979). Cell differentiation in isolated inner cell masses of mouse blastocysts *in vitro*: onset of specific gene expression. *J. Embryol. exp. Morph.* **53**, 367–379.

DZIADEK, M. & ADAMSON, E. D. (1978). Localization and synthesis of alphafoetoprotein in post-implantation mouse embryos. *J. Embryol. exp. Morph.* **43**, 289–313.

ENDERS, A. C. (1971). The fine structure of the blastocyst. In *The Biology of the Blastocyst* (ed. R. J. Blandau), pp. 71–94. Chicago: University of Chicago Press.

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. (1978*a*). Developmental potency of normal and neoplastic cells of the early mouse embryo. In *Proceedings of the Fifth International Conference on Birth Defects, Montreal 1977* (ed. J. W. Littlefield & J. de Grouchy; co-ed. F. J. G. Ebling), pp. 153–166. Amsterdam: Elsevier.

GARDNER, R. L. (1978*b*). 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* (ed. 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 tissue in the mouse. In *International Review of Experimental Pathology* Vol. 24 (ed. 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.* **80**, 251–288.

GARDNER, R. L. & PAPAIOANNOU, V. E. (1975). Differentiation in the trophectoderm and inner cell mass. In *The Early Development of Mammals: Second Symposium of the British Society of Developmental Biology* (ed. M. Balls & A. E. Wild), pp. 107–132. Cambridge: Cambridge University Press.

GARDNER, R. L. & ROSSANT, J. (1979). Investigation of the fate of 4·5 day *post-coitum* mouse inner cell mass cells by blastocyst injection. *J. Embryol. exp. Morph.* **52**, 141–152.

GARDNER, R. L., PAPAIOANNOU, V. E. & BARTON, S. C. (1973). Origin of the ectoplacental cone and secondary giant cells in mouse blastocysts reconstituted from isolated trophoblast and inner cell mass. *J. Embryol. exp. Morph.* **30**, 561–572.

GRAHAM, C. F. (1977). Teratocarcinoma cells and normal mouse embryogenesis. In *Concepts in Mammalian Embryogenesis* (ed. M. I. Sherman), pp. 315–394. Cambridge, Massachusetts: MIT Press.

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

HOGAN, B. & TILLY, R. (1977). *In vitro* culture and differentiation of normal mouse blastocysts. *Nature* **265**, 626–629.

LU, Y. L., SOBIS, H., VAN HOVE, L. & VANDEPUTTE, M. (1984). Differentiation potentiality of rat visceral yolk sac in organ culture. *J. Embryol. exp. Morph.* **80**, 127–136.

MCLAREN, A. & BOWMAN, P. (1973). Genetic effects on the timing of early development in the mouse. *J. Embryol. exp. Morph.* **30**, 491–500.

MCREYNOLDS, H. D. & HADEK, R. (1972). A comparison of the fine structure of late mouse blastocysts *in vivo* and *in vitro*. *J. exp. Zool.* **182**, 95–118.

NADIJCKA, M. & HILLMAN, N. (1974). Ultrastructural studies of the mouse blastocyst substages. *J. Embryol. exp. Morph.* **32**, 675–695.

NAESLUND, G., LUNDKVIST, O. & NILSSON, B. (1980). Transmission electron microscopy of mouse blastocysts activated and growth-arrested *in vivo* and *in vitro*. *Anat. Embryol.* **159**, 33–49.

NICHOLS, J. & GARDNER, R. L. (1984). Heterogeneous differentiation of external cells in individual isolated early mouse inner cell masses in culture. *J. Embryol. exp. Morph.* **80**, 225–240.

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.

PEDERSEN, R. A., SPINDLE, A. I. & WILEY, L. M. (1977). Regeneration of endoderm by ectoderm isolated from mouse blastocysts. *Nature* **270**, 435–437.

PETERSON, A. C., FRAIR, P. M. & WONG, G. G. (1978). A technique for detection and relative quantitative analysis of glucosephosphate isomerase isozymes from nanogram tissue samples. *Biochem. Genet.* **16**, 681–690.

REINIUS, S. (1965). Morphology of the mouse embryo from the time of implantation to mesoderm formation. *Z. Zellforsch. mikrosk. Anat.* **68**, 711–723.

ROSSANT, J. & OFER, L. (1977). Properties of extra-embryonic ectoderm isolated from postimplantation mouse embryos. *J. Embryol. exp. Morph.* **39**, 183–194.

SNELL, G. D. & STEVENS, L. C. (1966). Early embryology. In *Biology of the Laboratory Mouse, 2nd ed.* (ed. E. L. Green), pp. 205–245. New York: McGraw-Hill.

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

SOLTER, D., DAMJANOV, I. & SKREB, N. (1970). Ultrastructure of mouse egg-cylinder. *Z. Anat. EntwicklGesch.* **132**, 291–298.

SPIELMANN, 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.* **230**, 52–54.

WEST, J. D., BUCHER, T., LINKE, I. M. & DUNNWALD, M. (1985). Investigation of variability among mouse aggregation chimaeras and X-chromosome inactivation mosaics. *J. Embryol. exp. Morph.* **00**, 00–00.

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

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