J. Embryol. exp. Morph. Vol. 45, pp. 107–121, 1978
Printed in Great Britain © Company of Biologists Limited 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

By BRIGID HOGAN[1] AND RITA TILLY[1]

From the Imperial Cancer Research Fund, Mill Hill Laboratories, London

SUMMARY

This paper describes the development in culture of inner cell masses isolated immunosurgically from C3H/He mouse blastocysts immediately after collection between 3·5 and 4·0 days *p.c.* By 24–48 h most of the inner cell masses isolated from half-expanded blastocysts, and about 50 % of those from expanded blastocysts, regenerate an outer layer of trophectoderm-like cells and so resemble mini-blastocysts. With further *in vitro* culture these structures attach to the substratum and give rise to trophoblast-like giant cells, together with clusters of parietal endoderm cells or inner cell masses surrounded by visceral endoderm.

Many of the inner cell masses from the remaining expanded blastocysts develop into floating structures with an outer layer of endoderm cells, and by 7 days consist of a large fluid filled cyst surrounding a collapsed vesicle of epithelial cells. Mesodermal cells line the cysts and form numerous blood islands. When mechanically disrupted, and grown as attached sheets of cells, these cystic structures give rise to patches of trophoblast-like giant cells similar to those described in the previous paper.

These results suggest that the inner cell mass of normal mouse blastocysts contains cells which are capable of giving rise to trophoblast in culture.

INTRODUCTION

In the preceding paper we showed that inner cell masses isolated immunosurgically from fully expanded substage 3 and 4 mouse blastocysts can develop *in vitro* into structures similar to normal 7·5-day *p.c.* embryos, with embryonic ectoderm, embryonic and extra-embryonic mesoderm and visceral endoderm. They also contain a population of cells morphologically like the extra-embryonic ectoderm of the normal embryo, and when mechanically disrupted and grown as attached clumps give rise to cells resembling secondary trophoblast giant cells. Since the ICMs were isolated from fully expanded 4·5-day *p.c.* blastocysts it was possible that the extra-embryonic ectoderm-like cells were derived from some polar trophectoderm cells which had become internalized in the process of ectoplacental cone formation before immunosurgery rather than from cells of

[1] *Authors' address*: Imperial Cancer Research Fund, Mill Hill Laboratories, Burtonhole Lane, London, NW7 1AD, U.K.

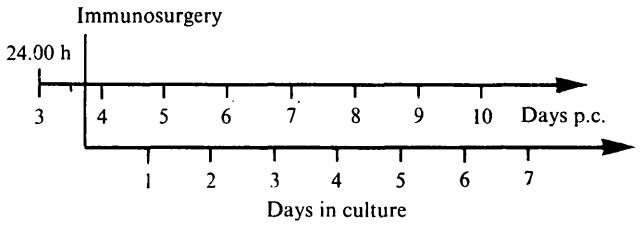


Fig. 1. Relative timing of immunosurgery and *in vitro* culture.

the ICM itself. By slight modification of our immunosurgery procedure we were able to isolate ICMs from earlier blastocysts in which there was very little possibility that polar trophectoderm had become internalized before immunosurgery. In this study we describe the *in vitro* development of these isolated inner cell masses.

METHOD

Blastocysts were collected from the uteri of C3H/He normally mated females as described in the preceding paper. Immunosurgery and *in vitro* culture were carried out according to the time schedule shown in Fig. 1. Zonae were removed from the blastocysts with a brief exposure to acidic Tyrode's solution at pH 2·5 containing 0·4 % (w/v) polyvinylpyrrolidone.

Immunosurgery and in vitro *culture*

In the initial experiments we found that our immunosurgery procedure A described in the preceding paper could not be used with blastocysts collected on the fourth day of pregnancy because all the cells, both trophectoderm and ICM, lysed on exposure to complement. Control experiments showed that this was due to antimouse antibodies in the guinea-pig serum which rendered it cytotoxic to 3·5-day *p.c.* ICM cells at 1:10 dilution. Method B was therefore introduced, in which the guinea-pig serum (collected fresh from ICRF bred animals and stored in aliquots at −70 °C) was diluted 1:50 to reduce its cytoxicity. This was finally superseded by Method C in which all anti-mouse activity in the guinea-pig serum was removed by prior absorption with agarose (Cohen & Schlesinger, 1970).

Method B

Embryos were transferred to 3 ml rabbit anti-mouse serum diluted 1:30 with DMEM supplemented with 10 % heat inactivated foetal calf serum and 5 mM HEPES buffer pH 7·3, and incubated for 10 min at 37 °C. After two washes in 3 ml of the supplemented DMEM they were transferred to 3 ml guinea-pig serum diluted 1:50 with supplemented DMEM and incubated for 30 min at 37 °C. This dilution of serum complement lysed all of the outer trophectoderm cells and was two times the minimum effective dilution (tested with a 1:30 dilution of antiserum).

Method C

As above, except that guinea-pig serum diluted 1:3 with DMEM and pre-absorbed with agarose before storing in aliquots at −70 °C was used without further dilution as a source of complement. With both methods, the same results were obtained if the incubation time in the rabbit antimouse serum was increased to 30 min.

After one wash in supplemented DMEM the embryos were incubated either in batches in 3 ml of DMEM plus 20 % HCS in 35 mm tissue culture dishes, or individually in a few microlitres of the same medium in wells of a micro test plate (Nunc). All incubations were at 37 °C in a humidified air/CO_2 incubator. The dead trophectoderm shells were not removed before culture; control experiments showed that they had no effect on the growth of the inner cell masses and in the batch cultures they prevented the embryos from aggregating and were shed spontaneously after 24 h.

Cell counts

The number of cells in blastocysts and isolated inner cell masses from which the dead trophoblast has been removed by pipetting was determined by the technique of Tarkowski (1966). The results were as follows: Blastocysts collected at 1.30 p.m. ± 30 min: half-expanded, 62 (range 52–67, 11 embryos counted); expanded, 62 (range 56–68, 9 embryos counted). Blastocysts collected at 5.30 p.m. ± 30 min: half-expanded, 67 (range 62–71, 6 embryos counted); expanded, 72 (range 62–107, 10 embryos counted). Inner cell masses isolated from pooled blastocysts collected at 1.30 p.m. ± 30 min: 18 (range 11–27, 9 ICMs counted). Inner cell masses from blastocysts collected at 5.30 p.m. ± 30 min: 22 (range 13–33, 8 ICMs counted).

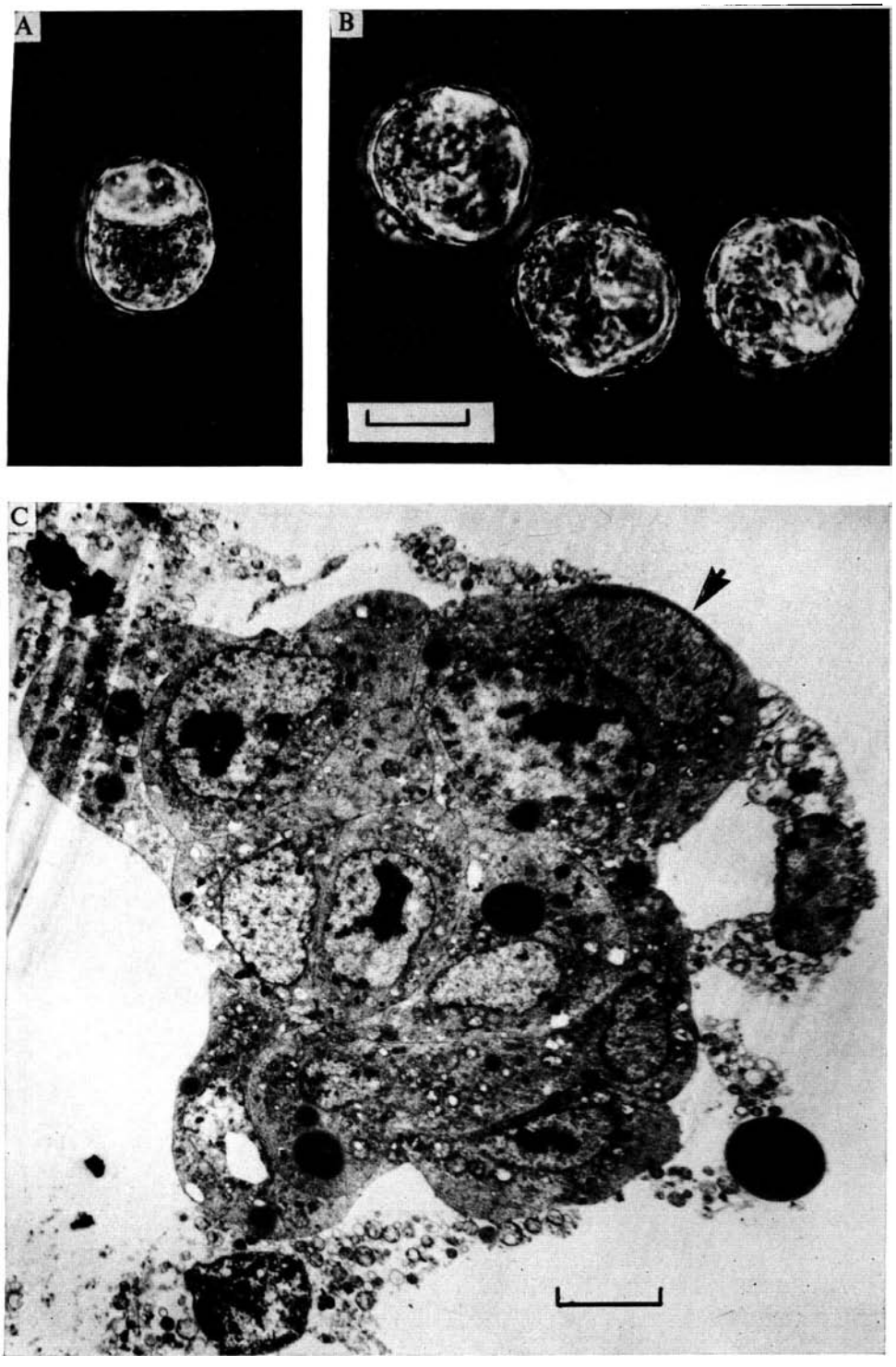
Light and electron microscopy

The fixation, embedding, sectioning and staining of embryos is described in the preceding paper.

RESULTS

In control experiments to test the efficiency of the immunosurgery procedure B with early blastocysts, a batch of embryos collected at 14.30 h on the 4th day of pregnancy was fixed immediately after the final incubation in complement, and serially sectioned. Figure 2C shows that the trophectoderm cells above the inner cell mass were clearly dead. In control blastocysts of this age we have found that the trophectoderm layer is often very thin over some inner cell mass cells; this may account for some cells not being entirely covered by remnants of dead trophectoderm after immunosurgery (see arrow in Fig. 2C).

In all expanded blastocysts collected up to 18.00 h and examined in the electron microscope there were no cells which could be unequivocally classified


A
B
C

as endoderm, as judged by the amount of endoplasmic reticulum, surface microvilli, and type of intercellular junctions which they displayed. In all cases the polar trophectoderm appeared to be a monolayer of cells.

In vitro *culture of inner cell masses isolated from blastocysts collected between 13.00 and 18.00 h on the 4th day of pregnancy*

Blastocysts collected at different times between 13.00 and 18.00 h (approx. 3·5–3·75 days *p.c.*) were divided into two classes; those in which the blastocoele cavity appeared by phase contrast microscopy to occupy 50 % or less of the total volume of the blastocysts (Fig. 2A, half-expanded blastocysts) and those in which the blastocoele cavity had expanded to occupy most of the total volume of the blastocyst (Fig. 2B, expanded blastocysts). Parallel electron microscope studies showed that these corresponded approximately to substages 1 and 2 of Nadijcka & Hillman (1974) respectively. Immunosurgery was then carried out as described in Methods, and the inner cell masses were incubated either in batches or singly. Results of typical experiments are given in Table 1. After 24–48 h incubation most of the inner cell masses isolated from half expanded blastocysts, and about 50 % of those from expanded blastocysts, have a striking resemblance to normal expanded blastocysts, with an outer trophectoderm-like wall surrounding an inner cluster of cells. Over the next few days these blastocyst-like structures continue to grow (Fig. 3A–D) and many of them either aggregate together in suspension or, more usually, attach to the tissue culture dish. The attached embryos show a variable degree of development; some only give rise to a few trophoblast giant cells and rounded cells which eventually disintegrate (Fig. 3B, C), while others behave more like normal blastocysts in culture and develop a sheet of trophoblast giant cells to which an inner cell mass surrounded by endoderm is attached (Fig. 3D). This inner cell mass may detach, giving rise to floating Type I structures (see preceding paper) in batch cultures. The internal organization of several blastocyst-like structures after 3–4 days in culture is shown in more detail in Fig. 4.

Electron microscope studies (Fig. 5A, B) confirm that the cells of the trophectoderm-like vesicles do indeed morphologically resemble trophectoderm cells of normal blastocysts (Enders, 1971). The cells are joined laterally by characteristic desmosomes and interdigitating membranes, and they contain

---

Fig. 2. Blastocysts before and after immunosurgery. A and B are phase-contrast pictures of blastocysts from one batch collected at 17.30 h on the 4th day of pregnancy (approx. 3·75 days *p.c.*). In A the blastocoele cavity occupies approximately 50 % of the total volume of the blastocyst (half-expanded); in B the blastocysts are expanded. C is an electron micrograph of a section through a blastocyst collected at 14.30 h and fixed immediately after immunosurgery (Method B). The outer trophectoderm layer is clearly dead. Bar is 50 μm for A and B and 10 μm for C.

Table 1. *Fate of inner cell masses isolated from blastocysts of different ages and cultured* in vitro. *In experiments marked with an asterisk the embryos were cultured individually in wells of a micro test plate*

Time embryos collected on the 4th day of pregnancy	Approx. days *p.c.*	Extent of blastocoele expansion	Days in culture after immuno-surgery	Type of structure formed in culture: Blastocyst-like (attached and un-attached)†	I‡	II§	IIIa‖	IIIb¶	IIIc††	Total
1 p.m.	3·5	25–50 %	3	32	7	—	—	—	—	39
5 p.m.	3·7	Expanded‡‡	7	8	—	—	9	5	—	22
5 p.m.*	3·7	Half-expanded‡‡	7	11	—	—	—	—	—	11
		Expanded	7	42	8	—	7	10	—	67
5·30 p.m.*	3·75	Half-expanded	7	3	—	—	—	—	—	3
		Expanded	7	13	6	—	1	2	—	22
6 p.m.	3·75	Expanded	7	10	—	—	7	18	3	38
11.30 p.m.	4·0	Expanded	5	5	3	—	—	—	11	19

† See Fig. 3A–D. ‡ See fig. 4B, Hogan & Tilly, 1978. § See Fig. 6A. ‖ See text. ¶ See Fig. 8A, B.
†† See fig. 6, Hogan & Tilly, 1978. ‡‡ See Fig. 2

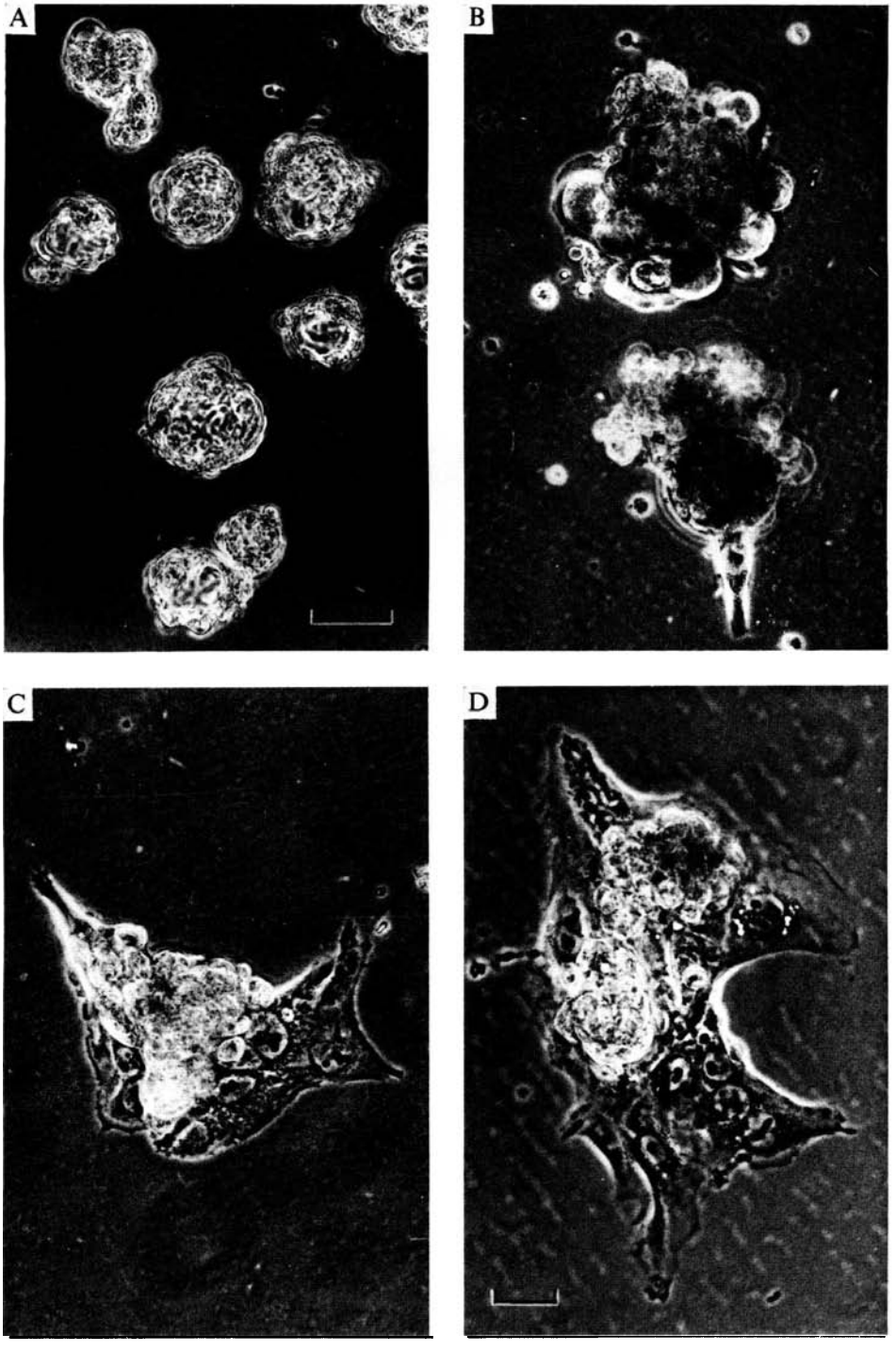


Fig. 3. Phase-contrast microscopy of inner cell masses isolated from half-expanded 3·7-day blastocysts and cultured *in vitro*. (A) After 3 days in culture. Floating structures resembling normal expanded blastocysts. (B–D) After 7 days in culture. Blastocyst-like structures which have attached to the tissue culture dish. A–C were derived from ICMs isolated using immunosurgery method B. In D, immunosurgery method C was used. Bar is 100 μm for A and 50 μm for B, C and D.

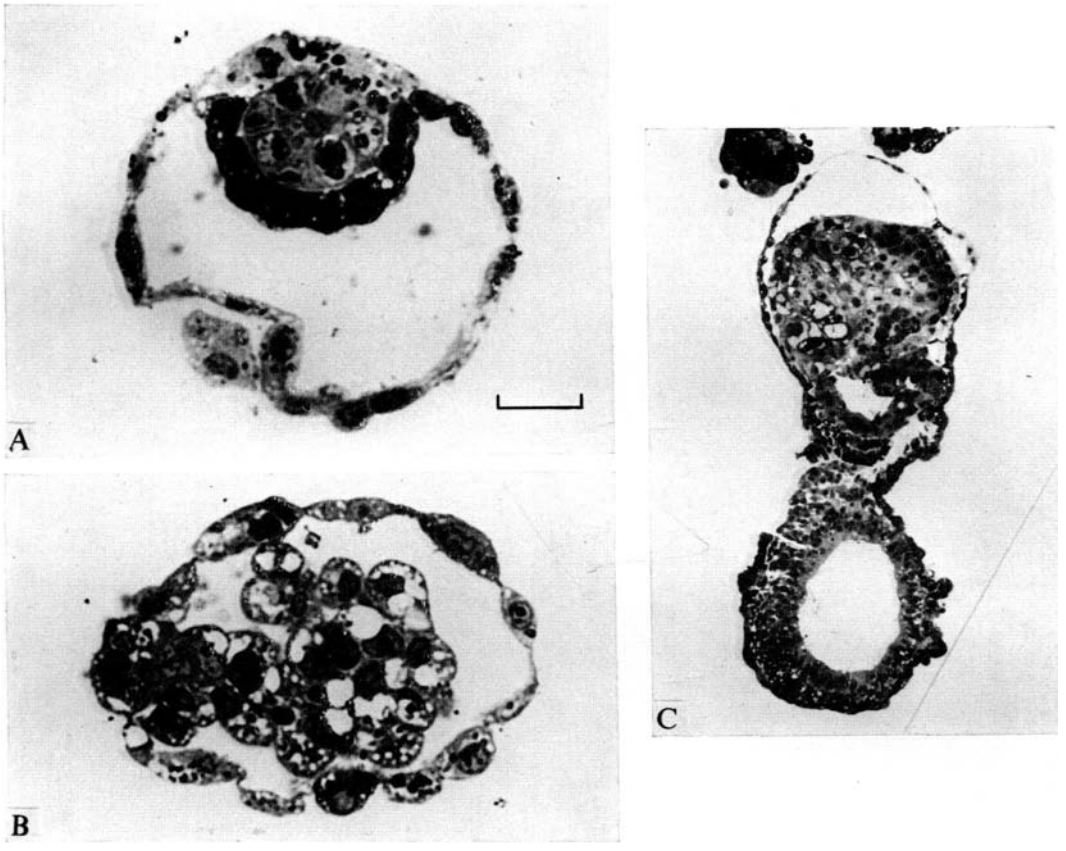


Fig. 4. Sections through blastocyst-like aggregates developing from inner cell masses isolated from half-expanded 3·7-day blastocysts and cultured *in vitro*. (A), (B) After 3 days in culture. A closely resembles a normal late blastocyst, while in B a vesicle of trophectoderm-like cells surrounds a cluster of highly vacuolated cells. (C) After 4 days in culture. There has been further proliferation of inner cell mass and trophectoderm cells. Bar is 20 μm for A and B and 100 μm for C.

numerous glycogen granules and vacuoles, and have few microvilli on the outer surface.

Electron microscopy also shows that the highly vacuolated cells seen in some aggregates (for example, Fig. 4B) contain swollen endoplasmic reticulum filled with secretory material and are often surrounded by sheets of basement membrane. They therefore resemble highly vacuolated parietal endoderm cells of normal embryos.

As shown in Table 1, the remainder of the inner cell masses isolated from expanded 3·7 to 3·75-day *p.c.* blastocysts do not regenerate blastocyst-like structures but form floating structures surrounded by an outer layer of visceral endoderm cells. The early development of these cultured ICMs is very similar to that described in the preceding paper (Hogan & Tilly, 1978) for inner cell

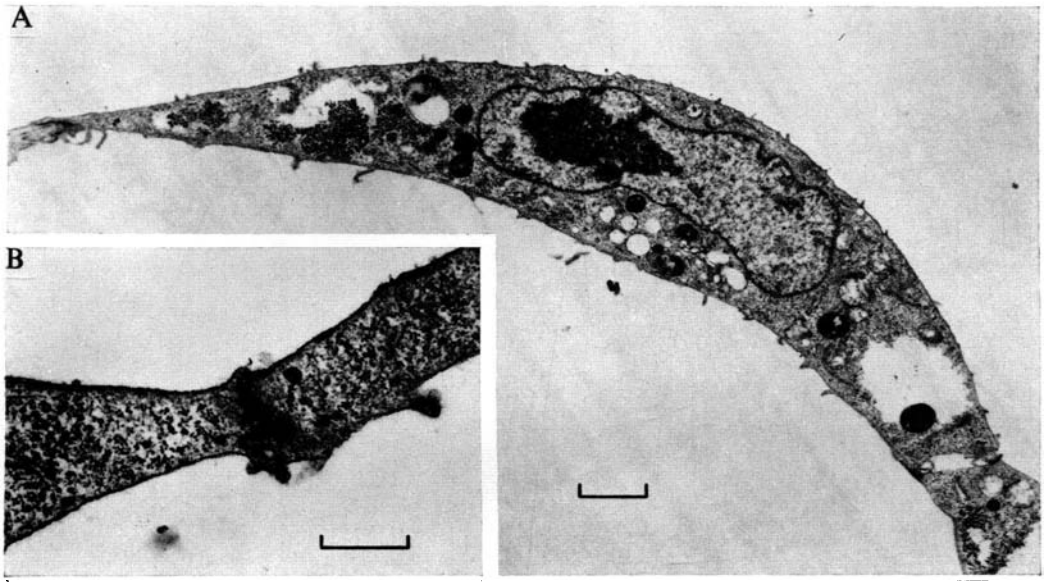


Fig. 5. Trophoblast-like cells formed after 3 days in culture. (A) Typical cell forming part of a trophectoderm-like vesicle. (B) Detail of a junction between two adjacent trophoblast-like cells. Bar is 2·5 μm in A and 0·5 μm in B.

masses isolated from older blastocysts, but proceeds more slowly, so that Type I and Type II structures are not observed until about day 3 and day 5 of culture respectively. Two typical Type II cultured ICMs showing the characteristic thin cells in the inner vesicle are displayed in Fig. 6A. From sections (Fig. 6B–D) it is apparent that mesodermal-like cells arise in exactly the same way as described in the preceding paper (Hogan & Tilly, 1978), namely by delamination from a layer of columnar epithelial cells close to a junction with the hemisphere of elongated cells. The mesodermal-like cells also appear to migrate between the inner cells and the overlying endoderm.

By 7 days in culture two new kinds of structures are present. Some consist of a hollow vesicle of epithelial cells, separated from the outer endoderm cells by a loose network of mesenchymal cells. These are classified as Type IIIa structures in Table I. The other type (IIIb) consists of a very large fluid filled cyst (up to 2 mm in diameter) surrounding a small collapsed vesicle of epithelial cells on one side. The internal structure of two typical Type IIIb structures is shown in detail in Fig. 7A and B. A continuous layer of mesenchymal cells extends over the vesicle of epithelial cells and underneath the outer endoderm layer, where it becomes elaborated into blood islands containing nucleated, pigmented erythroblasts. In some cases the mesenchymal cells form an allantois-like structure (Fig. 7A). The disorganized epithelial vesicle is made up of cells with a variety of shapes and sizes, ranging from elongated to rounded and columnar. All of them are bounded on the outer surface by a thin, discrete

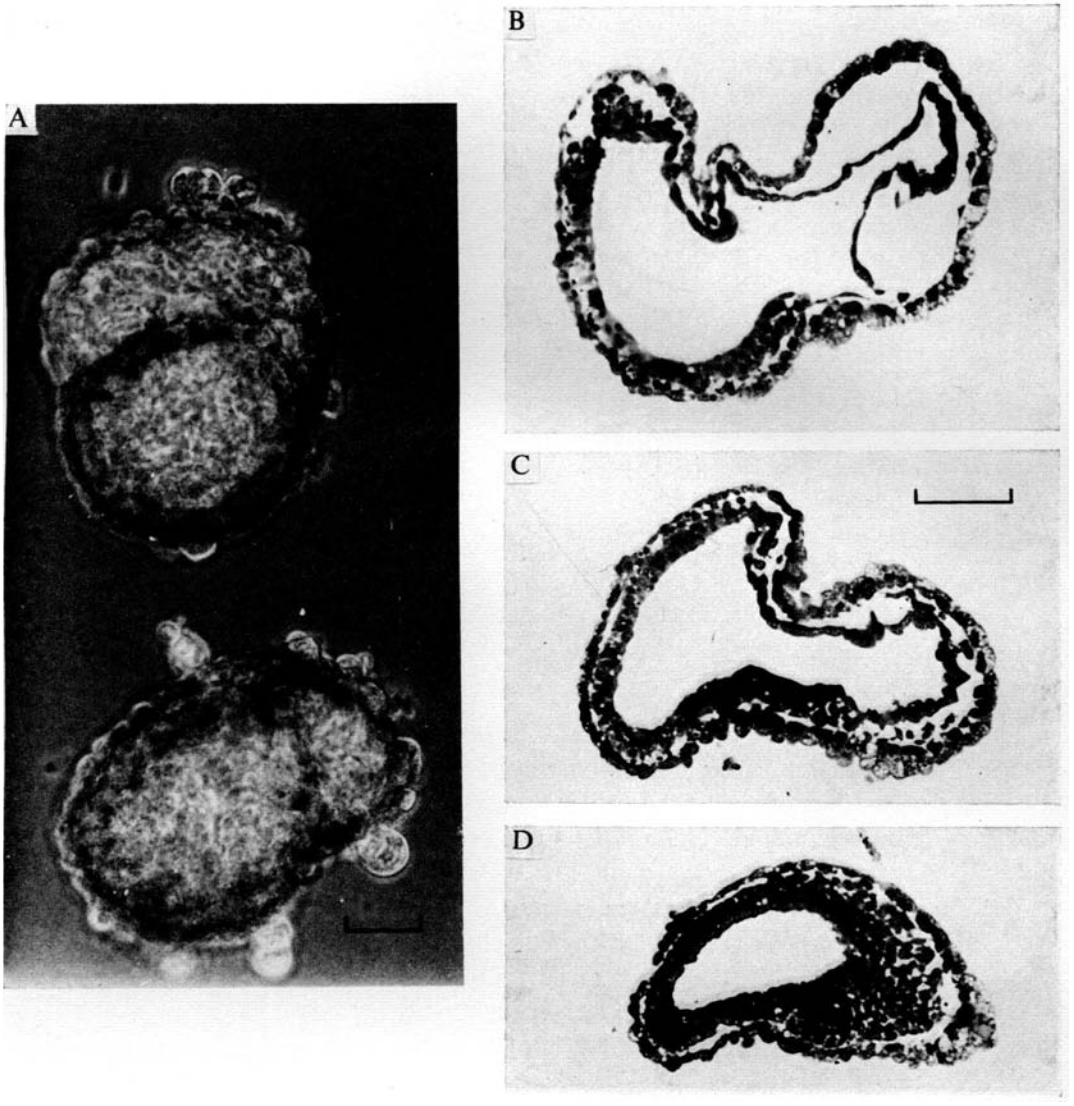


Fig. 6. Type II structures which have developed from inner cell masses isolated from expanded blastocysts collected at 18.00 h (A) and 17.20 h (B) on the 4th day of pregnancy. (A) After 3 days in culture. Phase-contrast microscopy. The thin cells of the inner vesicle are clearly detached from the overlaying endoderm cells. (B–D) Three sections taken at different levels in the same cultured ICM, which was fixed 5 days after immunosurgery. Mesodermal cells are present in C and D, and elongated epithelial cells in B and C. Bar is 50 μm.

basement membrane, and have little endoplasmic reticulum and some microvilli. When Type IIIb structures formed after 7 days in culture are disrupted mechanically and incubated in DMEM plus 20 % HCS, clumps of cells attach to the surface of the dish and a variety of cell types, including beating muscle, appear

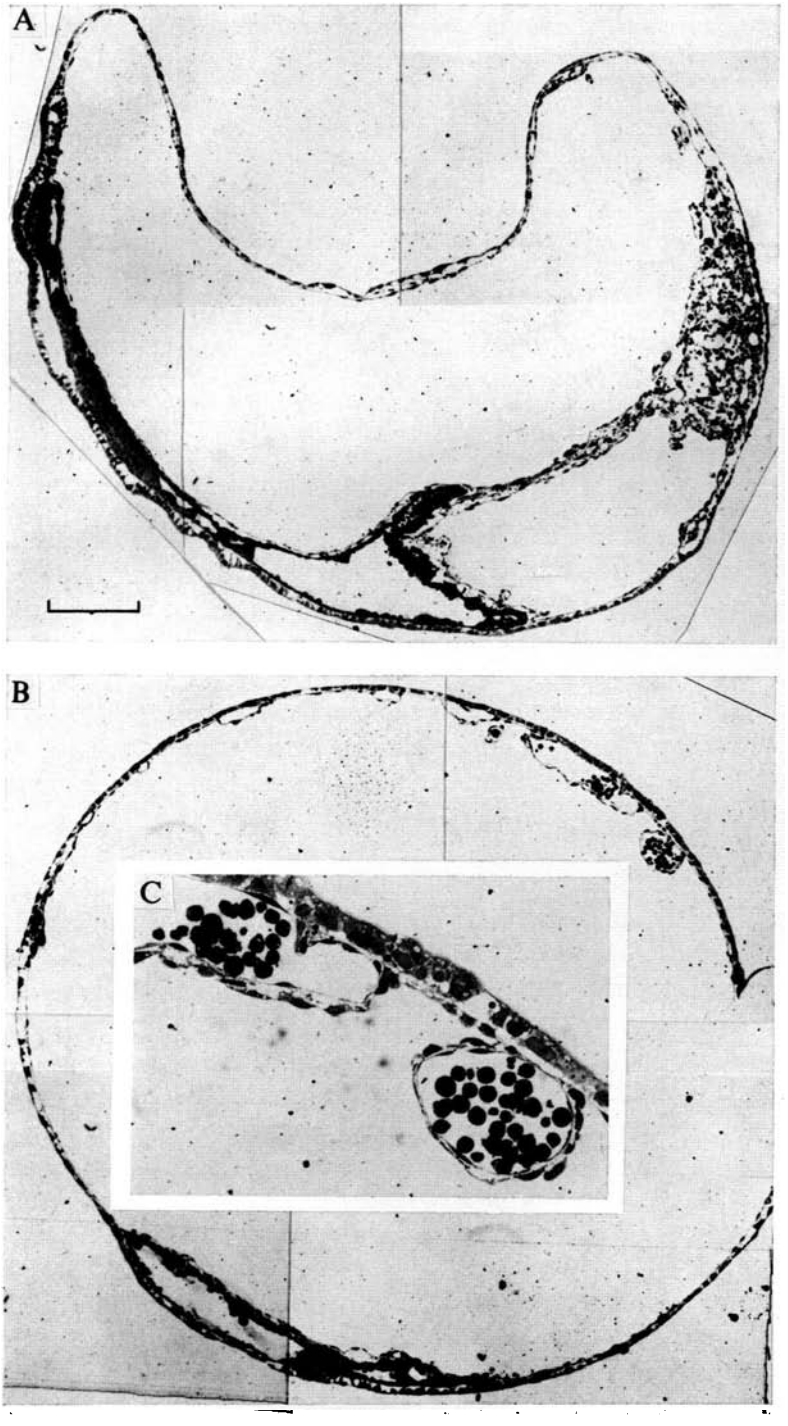


Fig. 7. Sections through Type IIIb cultured ICMs fixed after 7 days in culture. In B, blood islands are present in the mesodermal layer beneath the outer endoderm layer. Details of two islands are shown in C. Bar is 200 μm in A and B and 50 μm in C.

in the culture. Large patches of cells similar to the trophoblast giant cells shown in fig. 7 of the preceding paper (Hogan & Tilly, 1978) also appear after a few days.

In vitro *culture of inner cell masses isolated from blastocysts collected at 23.00 h on 4th day of pregnancy*

A batch of 19 embryos collected at 23.00 h consisted of expanded blastocysts, and some blastocysts which had hatched from their zonae and had large mural trophectoderm cells. About half of the inner cell masses isolated from these blastocysts developed within 5 days into structures which resembled normal 7·5-day embryos with embryonic ectoderm, embryonic and extra-embryonic mesoderm, and a chorion-like structure, all surrounded by visceral endoderm (see fig. 6 of the preceding paper (Hogan & Tilly, 1978)).

DISCUSSION

In this paper we show that most of the inner cell masses isolated immunosurgically from half-expanded 3·5- to 3·75-day *p.c.* blastocysts, and about 50 % of those from expanded blastocysts, regenerate blastocyst-like structures when incubated *in vitro* for 24–48 h. These structures contain cells morphologically identical to trophectoderm, and some later give rise to sheets of trophoblast-like giant cells and inner cell masses surrounded by visceral endoderm.

There are several possible explanations for the regeneration of trophectoderm by inner cell masses isolated from early blastocysts. Firstly, the immunosurgery procedure may not kill all the outer trophectoderm cells. We consider this possibility very unlikely (both for Method B and, in particular, for Method C) for the reasons outlined in the preceding paper (Hogan & Tilly, 1978). In addition, virtually all of the inner cell masses from half-expanded blastocysts regenerate trophectoderm vesicles (Table 1), and it seems hardly likely that unkilled trophectoderm cells persisted on the surface of every one of these inner cell masses.

A second possibility, which we favour, is that for some time after differentiation of the morula into a blastocyst, some or all of the cells in the inner cell mass are able to reverse their fate and form trophectoderm cells when they are exposed to 'outside' conditions. If this is correct, such reversal of fate seems to be possible with inner cell masses from C3H/He embryos up to about 3·7–3·75 days *p.c.*, i.e. around the time when primary endoderm formation may begin. Handyside (Handyside, 1977; Johnson, Handyside & Braude, 1977) has reported that inside cells isolated immunosurgically from late morula and early blastocysts (3·2–3·3 days *p.c.*) of CFLP mice are able to regenerate a trophectoderm layer, while inside cells from expanded (3·5 days *p.c.*) blastocysts form only an outer layer of endoderm. This suggests that the exact time at which inner cells become restricted in their fate and committed to forming endoderm may vary from one mouse strain to another. Recent work by Johnson

& Handyside (1977) has shown that even when the fate of the inner cells is still reversible they appear to be synthesizing new proteins not made at an earlier stage of development. This suggests that synthesis of a new set of proteins (and therefore possibly activation of a new set of genes) does not *per se* preclude reversal of cell fate. This idea is supported by studies on the cellular slime mould *Dictyostelium discoideum*. Soll & Waddell (1975) showed that even 10–14 h after the onset of the aggregation programme, when many changes have occurred in the synthesis of specific enzymes (reviewed by Loomis, 1975), *Dictyostelium* cells are able to reverse their morphological and biochemical differentiation and form free-living amoebae if they are dissociated and suspended in nutrient medium. The time required for erasure of their morphogenetic programme is longer for cells dissociated at 14 rather than at 10 h (approximately 200 and 100 min respectively) and reversal is prevented by inhibiting protein synthesis. The fact that time is required to reverse cell fate may account for the reported failure of inner cell mass cells isolated microsurgically from 3·5-day blastocysts to give rise to trophoblast derivatives when aggregated with 2·5 days *p.c.* morula cells (Rossant, 1975). It could be argued that by the time any accumulated biochemical changes in the inner cell mass cells were reversed, the morula cells had already formed all the trophectoderm that was needed.

In contrast to inner cell masses from earlier blastocysts, those isolated from about 50 % of expanded 3·7- to 3·75-day *p.c.* blastocysts and nearly all 4·0-day *p.c.* blastocysts behave essentially as described in the preceding paper (Hogan & Tilly, 1978). The difference in the internal organization of Type IIIb and c cultured ICMs is probably not significant. Both types are preceded by structures which have a very similar internal organization (see fig. 5, Hogan & Tilly, 1978; Fig. 6 in this paper). In this intermediate stage, mesodermal-like cells appear to delaminate from a layer of columnar epithelial cells close to where it forms a junction with a hemisphere of elongated cells. If the vesicle of columnar and elongated cells were to collapse soon after mesoderm formation, then a Type IIIb structure would develop. If, on the other hand, the vesicle of inner cell remains expanded (possibly because the cells are growing more rapidly) then a Type IIIc structure would develop, with highly organized layers of embryonic ectoderm and chorion, arising from the elongated cells. Moreover, both Type IIIb and c structures give rise to trophoblast-like giant cells when mechanically disrupted and grown as attached sheets.

The possible explanations for the fact that isolated inner cell masses from 3·7 to 4·0-day *p.c.* blastocysts can apparently give rise to trophoblast derivatives, either in the form of well organized extra-embryonic ectoderm tissues or as attached sheets of trophoblast-like giant cells, are the same as those outlined in the preceding paper (Hogan & Tilly, 1978). However, one possibility, that some trophoblast cells had become internalized in the process of ectoplacental cone formation before immunosurgery, is very unlikely for blastocysts at this stage of development, and we have seen no morphological evidence for it.

This leaves two likely hypotheses; that the extra-embryonic ectoderm of the normal embryo is of dual origin, or that some cells of the inner cell mass remain pluripotent and able to regenerate trophectoderm derivatives when placed in the right environment. Again, we are at present unable to distinguish between these two possibilities.

The results presented in these two papers may tell us something about the way in which early mammalian development is controlled. For example, they make it unlikely that the key events in early development proceed by groups of cells making immediately irreversible decisions between alternative differentiation programmes (patterns of gene expression). One such key event is the formation of a blastocyst from a group of about 16 morula cells. If it is assumed that all of the morula cells are equipotent – and there is good evidence that this is true for the 8-cell stage (Kelly, 1977) – then differentiation into two morphologically distinct classes of cell, the outer trophectoderm and the inner cell mass, might proceed in several possible ways. For example, at one particular time all of the cells might make an irreversible choice, depending on their position, to be either trophectoderm or inner cell mass. Alternatively, only the outer cells might make such an irreversible decision and the inner cells remain potentially capable of altering their fate until, perhaps under the influence of the outside cells, they too eventually make an irreversible decision. Thirdly, both the inside and the outside cells may be able to reverse their differentiation programmes for some time. Our finding, and that of Handyside (Handyside, 1977; Johnson *et al.* 1977) that inner cells from early blastocysts (up to about 3·5–3·7 days *p.c.* depending on strain) are able to regenerate trophectoderm in culture is incompatible with the first model, but is compatible with either of the other two.

In the above discussion we have only considered the situation in which all of the inner cell mass cells are potentially capable of reversing their fate and forming trophectoderm. However, there is also the possibility that only a subpopulation of inner cells remains pluripotent throughout early development, and these could conceivably be destined to give rise to the totipotent germ cells.

We thank Gillian McArthur, James Vernon, Frank Fitzjohn and the staff of the SPF breeding unit for skilled technical assistance.

REFERENCES

COHEN, A. & SCHLESINGER, M. (1970). Absorption of guinea pig serum with agar. *Transplantation* **10**, 130–132.

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

HANDYSIDE, A. H. (1977). Time of commitment of inside cells isolated from preimplantation mouse embryos. *J. Embryol. exp. Morph.* **45**, 37–53.

HANDYSIDE, A. H. & JOHNSON, M. H. (1977). Temporal and spatial patterns of the synthesis of tissue-specific polypeptides in the preimplantation mouse embryo. *J. Embryol. exp. Morph.* **44**, 191–199

HOGAN, B. & TILLY, R. (1978). *In vitro* development of inner cell masses isolated immunosurgically from mouse blastocysts. I. Inner cell masses from 3·5-day *p.c.* blastocysts incubated for 24 h before immunosurgery. *J. Embryol. exp. Morph.* **45**, 93–105.

JOHNSON, M. H., HANDYSIDE, A. H. & BRAUDE, P. R. (1977). Control mechanisms in early mammalian development. In *Development in Mammals*, **2** (ed. M. H. Johnson), pp. 67–95. Amsterdam: North Holland.

KELLY, S. J. (1977). Studies of the development potential of 4- and 8-cell stage mouse blastomeres. *J. exp. Zool.* **200**, 365–376.

LOOMIS, W. F. (1975). *Dictyostelium discoideum. A Development System.* New York: Academic Press.

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

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

SOLL, D. R. & WADDELL, D. R. (1975). Morphogenesis in the slime mould *Dictyostelium discoideum*. I. The accumulation and erasure of 'morphogenetic information'. *Devl Biol.* **47**, 292–302.

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

(Received 13 September 1977, revised 12 December 1977)