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

By BRIGID HOGAN and RITA TILLY

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

SUMMARY

This paper describes the in vitro development of inner cell masses isolated immunosurgically from mouse blastocysts which had been collected on 3-5 days p.c. and then incubated for 24 h. The inner cell masses continue to grow in culture and develop through a series of stages with increasing complexity of internal organization. By day 1 all of the cultured ICMs have an outer layer of endoderm, and by day 3 some of them have two distinct kinds of inside cells; a columnar epithelial layer and a thin hemisphere of elongated cells. Later, mesodermal cells appear to delaminate from a limited region of the columnar layer, close to where it forms a junction with the thinner cells. By day 5, about 25% of the cultured ICMs have a striking resemblance to normal 7.5-day p.c. C3H embryos, with embryonic ectoderm, extra-embryonic ectoderm and chorion, embryonic and extra-embryonic mesoderm, and visceral endoderm. When mechanically disrupted and grown as attached clumps of cells in a tissue dish, these embryo-like structures give rise to trophoblast-like giant cells. These results suggest that the inner cell mass of 4-5-day p.c. blastocysts contains cells which can give rise to trophoblast derivatives in culture.

INTRODUCTION

The preimplantation mouse blastocyst consists of an outer layer of trophectoderm surrounding the blastocele cavity and inner cell mass. A technique known as 'immunosurgery' has recently been described (Solter & Knowles, 1975) for selectively killing the outer trophectoderm cells by exposing the blastocysts sequentially to antispecies antibody, washing medium and complement. Following immunosurgery, the isolated inner cells continue to multiply and differentiate in vitro for up to several weeks. This combination of immunosurgery and in vitro culture may provide information about the development potential of the inner cells at different times after blastocyst formation, and might be used to build up a picture of the timing and interrelationship of cell determination, morphogenesis and cell lineage in the mammalian embryo. In this and the

1 Authors' address: Imperial Cancer Research Fund, Mill Hill Laboratories, Burtonhole Lane, London, NW7 1AD, U.K.
subsequent paper we describe in detail the growth and morphogenesis of inner cell masses isolated from C3H/He blastocysts of different ages. For technical reasons, the initial experiments used blastocysts collected on 3-5 days p.c. and then incubated in vitro for 24 h before immunosurgery; the development of the inner cell masses from these blastocysts is the subject of this paper. The development of inner cell masses isolated from blastocysts collected between 3-5 and 4-0 p.c. is described elsewhere (Hogan & Tilly, 1978).

**METHODS**

Embryos were obtained from 8- to 12-week-old normally mated inbred C3H/He mice and cultured in vitro according to the schedule shown in Fig. 1. 12.00 h on the day on which plugs were first observed was considered to be 0-5 days post coitum (p.c.)

**Isolation of blastocysts**

Blastocysts were flushed from uteri between 14.00 and 16.00 h on the 4th day of pregnancy (approximately 3-6 days p.c.). Embryos from the same female varied in their stage of development; the majority appeared by phase contrast microscopy to be either substage 1 (early) or substage 2 (mid) blastocysts according to the classification of Nadijcka & Hillman (1974) (see also fig. 2, Hogan & Tilly, 1978). The zonae pellucidae were removed with a brief incubation at 37 °C in pronase (0.5 % (w/v) in PBS with 0.5 % polyvinylpyrroldone) and the blastocysts incubated 24 h in Dulbecco’s modified Eagle’s medium (DMEM) containing 20 % human cord serum (HCS, heat inactivated 56 °C for 30 min) in a humidified air/CO₂ incubator at 37 °C.

**Immunosurgery**

The antiserum used in all experiments was prepared by bleeding a rabbit 10 days after three fortnightly injections of 4 × 10⁸ C57BL spleen cells. The serum was heat inactivated at 56 °C for 30 min and was found to be effective in the immunosurgery procedure at dilution of up to 1:200.
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Table 1. Differentiation in vitro of immunosurgically isolated inner cell masses into structures of different classes, as judged by phase contrast microscopy

Examples of the three types of structure are shown in Fig. 3, and are described in the text. In A, which represents the combined results of three separate experiments, the dead trophectoderm was not removed after immunosurgery. In B, which represents the combined results of two separate experiments, the trophectoderm was removed by sucking the embryos into a finely drawn out Pasteur pipette.

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<td>(B) Dead trophectoderm removed after immunosurgery</td>
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Method A

Immunosurgery was routinely carried out between 14.00 and 16.00 h (equivalent to 4-6 days p.c.). Blastocysts, transferred in a few microlitres of medium, were incubated for 30 min at 37 °C in 3 ml of antiserum diluted 1:30 with DMEM containing 0.5 % bovine serum albumin (Sigma fraction V) or 10 % heat inactivated foetal calf serum. They were then washed twice in 3 ml of the same medium and transferred to 3 ml of guinea-pig complement (Wellcome freeze-dried guinea-pig serum, diluted 1:10). After 30 min at 37 °C the outer trophectoderm cells had all lysed and the embryos were washed twice in 3 ml supplemented DMEM. The final incubation was in 3 ml of DMEM plus 20 % HCS and the medium was changed every 3-4 days.

In contrast to Solter & Knowles (1975) we routinely did not remove the dead trophectoderm cells before incubating the embryos further. We found that the inner cell masses grew well inside their trophectoderm shells and were prevented by them from aggregating together and from attaching to the substratum; after about 20 h the inner cell masses separated from the shells spontaneously. Control experiments showed that similar results were obtained when the dead trophectoderm cells were removed by pipetting immediately after exposure to complement (see Table 1).

Light microscopy and electron microscopy

Embryos were placed in 2.5 % glutaradehyde buffered with 0.06 M sodium cacodylate at pH 7.3 for 1 h, rinsed in three changes of cacodylate buffer and post-fixed in 1 % osmium tetroxide buffered with veronal acetate at pH 7.3. Dehydration was carried out in a series of graded ethanols before embedding in Araldite medium.

For histological examination several sections each 1 μm thick were taken
Fig. 2. Typical blastocyst at the time of immunosurgery, showing a layer of endoderm cells on the blastocoele side of the inner cell mass. This corresponds to substage 3 of Nadijcka & Hillman (1974). In some blastocysts, endoderm cells had migrated along the trophectoderm (substage 4). Bar is 25 μm.

every 10–20 μm through the embryos. As the sections were cut on a Cambridge Ultramicrotome, thin sections could also be taken at these intervals for electron microscopy. The sections for light microscopy were mounted on glass slides and stained with 1 % toluidine blue in 1 % borax solution, and the sections for electron microscopy were mounted on copper grids and stained with uranyl acetate solution followed by lead citrate, before being examined in a Siemens Elmskop 1 electron microscope.

RESULTS

Immunosurgery of blastocysts

At the time when immunosurgery was routinely carried out (equivalent to 4 days 14 h p.c.) all of the embryos were fully expanded blastocysts, and in section had a layer of endoderm cells on the blastocoele side of the inner cell mass (Fig. 2). They corresponded to substage 3 or 4 blastocysts according to the classification of Nadijcka & Hillman (1974). Judging from electron microscope sections, there was no obvious proliferation of polar trophectoderm to form
Fig. 3. Phase-contrast microscopy of isolated inner cell masses at different times during in vitro incubation: (A) after 20 h, (B) after 48 h (Type I), (C) after 3 days (Types I and II), and (D) after 5 days (Type III). Bar is 100 μm in A–C and 200 μm in D.
Fig. 4. Sections through typical cultured ICMs at different times after immuno-
surgery. (A) After 48 h (Type I), (B) after 3 days (Type II), showing the two popu-
lations of inside cells; columnar epithelial and elongated. Bar is 50 μm.
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ectoplacental cone. In fourteen blastocysts fixed immediately after immunosurgery and serially sectioned at 1 μm, all the outer cells, including those above the inner cell mass, were clearly lysed, and the nuclear membranes disrupted.

Inner cell masses 20–24 h after immunosurgery

After 20 h in culture, immunosurgically isolated inner cell masses separate from their trophectodermal shells but remain floating and do not attach to the substratum (Fig. 3A). They consist of a solid core of cells surrounded by a monolayer of endoderm cells which is probably formed by the growth and spreading of the endoderm cells present before immunosurgery. A thin layer of finely fibrillar basement membrane material is present between the outer endoderm cells and the inner core; this basement membrane is presumably secreted by the overlying endoderm cells which have some rough endoplasmic reticulum, as well as microvilli, vacuoles and cytoplasmic inclusions.

Cultured inner cell masses 48 h after immunosurgery

After 48 h, most of the cultured ICMs have a central cavity within the epiblast (embryonic ectoderm) (Fig. 3B, 4A). This central cavity appears to be formed initially by cell death; in many instances dead cells and debris are present in the centre, while adjacent cells are completely healthy and have numerous microvilli on their free surface.

The cells of the outer endoderm layer, which in some cases have proliferated to form loosely attached clumps (Fig. 3B), now clearly resemble visceral endoderm cells of the normal embryo (Solter, Damjanov & Škreb, 1970) and the basement membrane remains thin.

Cultured inner cell masses 3 days after immunosurgery

After 3 days in culture all of the cultured ICMs have developed into hollow vesicles, but they show a variable degree of expansion (Fig. 3C). About 67% are only slightly larger than at 48 h and are classified as Type I structures in Table 1. The remainder are considerably more expanded and in the phase contrast microscope some of the inner cells are distinctly thinner than the rest; these are classified as Type II structures. As shown in Table 1, the proportion of the two types present on day 3 was not significantly affected by removing the dead trophectoderm cells after immunosurgery.

In sections of Type II structures there is clearly a subdivision of the inner vesicle into two regions; one in which the cells are packed into an epithelial layer, and another in which the cells are much more elongated (Fig. 4B). Apart from their shape, there is very little difference in the morphology of the cells in the two regions and they have few cytoplasmic organelles and little endoplasmic reticulum. On the side facing the proamniotic cavity the cells are joined apically by junctional complexes and they have some microvilli which are covered in a layer of extracellular material. Some of the elongated cells interdigitate along their adjoining surfaces, but this is not a regular feature.
Fig. 5. Section through a typical cultured ICM fixed 5 days after immunosurgery. The structure has collapsed during subsequent handling. The arrows show the junction between the columnar epithelial layer and the hemisphere of more rounded cells. Early mesoderm cells appear to be delaminating from a localized region near the junctional zone. Bar is 50 μm.
Fig. 6. Sections taken at two levels through a single Type III cultured ICM, fixed 5 days after immunosurgery. The open star marks the region equivalent to the ectoplacental cavity of the normal embryo, while the closed star denotes the equivalent of the exocoelom, which is continuous with the proamniotic cavity. Bar is 100 μm.

In all structures the outer cells resemble visceral endoderm of normal embryos, with numerous microvilli, vacuoles, membrane-bound degenerative inclusions and extensive endoplasmic reticulum. The basement membrane separating them from the inner cells remains thin.

**Cultured inner cell masses 5 days after immunosurgery**

Between 3 and 5 days after immunosurgery a number of striking changes take place in the internal organization of some of the larger cultured ICMs, so that by day 5 about 25% of them have a striking resemblance to the egg-cylinder of normal C3H/He embryos at about 7.5 days p.c., with cells which correspond in their morphology to embryonic and extra-embryonic ectoderm, embryonic and extra-embryonic mesoderm and visceral and embryonic endoderm (Solter et al. 1970). These are classified as Type III structures (Fig. 3D, 6A and B).

A possible intermediate stage between a Type II and Type III structure is shown in Fig. 5. The closely packed, columnar epithelial layer presumably corresponds to embryonic ectoderm, while the hemisphere of more rounded and lighter staining epithelial cells resembles extra-embryonic ectoderm of normal
Fig. 7. Giant trophoblast cells in culture. A batch of Type III cultured ICMs 5 days after immunosurgery was mechanically disrupted by sucking them into a finely drawn out Pasteur pipette. The clumps of cells were then incubated in a 35 mm tissue dish with DMEM plus 20% HCS. After 2 days, scattered patches of cells morphologically like trophoblast giant cells were present in the culture. They were often associated with large clusters of rounded, less adherent cells (centre). Bar is 100 μm.

embryos. The population of mesodermal-like cells seems to arise by delamination from the embryonic ectoderm, over a localized region near the junction with the presumed extra-embryonic ectoderm.

In more advanced stages (Fig. 6A, B) the mesoderm has apparently migrated between the embryonic and extra-embryonic ectoderm and overlaying endoderm and has formed structures equivalent to the amnion and exocoelom of the normal embryo. The embryonic ectoderm cells are bounded by a thin basement membrane, except at the region of the 'primitive streak', and the mesodermal cells always lie between this basement membrane and the endoderm cells. The presumed extra-embryonic ectoderm is made up of both rounded and more elongated cells which have few cytoplasmic organelles or microvilli, and are joined along extensive areas of adjacent cytoplasm by junctional complexes. In some embryos the hemisphere of extra-embryonic ectoderm-like cells becomes rolled up into a flattened, chorion-like vesicle surrounding an ectoplacental cavity. This is clearly shown in Fig. 6A and B which are sections at different levels through a single Type III cultured ICM. Of five other Type III
structures sectioned every 20 μm (see Methods), four resembled Fig. 6A all the way through, while one resembled Fig. 6B, with the chorion-like structure only being found in some sections.

Several distinct kinds of outer endoderm cells are visible. Adjacent to the junction between the embryonic and extra-embryonic ectoderm the endoderm cells are very large, and the cytoplasm is filled with sheets of narrow endoplasmic reticulum. At the base of the ‘egg-cylinder’, the endoderm cells are flattened and have fewer microvilli and less endoplasmic reticulum. Both kinds of endoderm closely resemble cells in equivalent positions in normal 7-5-day p.c. embryos (Solter et al. 1970). Cells resembling parietal endoderm have never been observed.

Further differentiation in vitro

When maintained in suspension culture the cultured ICMs do not develop much beyond the stages shown in Figs. 3D and 6A and B. However, if the embryo-like structures are mechanically disrupted by pipetting through a finely drawn out Pasteur pipette, clumps of cells attach to the substratum and after 3–5 days many different tissues are present, including beating muscle, red blood cells and nerve. In addition, as shown in Fig. 7, large patches of cells appear which closely resemble the giant trophoblast cells which develop when extra-embryonic ectoderm from normal 9-day embryos is cultured in vitro (Rossant & Ofer, 1977).

DISCUSSION

One of the striking features of these results is the high degree of organization and differentiation which can be achieved in culture by individual inner cell masses isolated immunosurgically from fully expanded blastocysts. On average about 25 % of the inner cell masses develop within 5 days into structures which closely resemble normal 7-5-day p.c. embryos. Further incubation has little effect on the number of cultured ICMs showing this degree of development, and many never progress beyond a simple hollow vesicle surrounded by endoderm. This failure to develop is probably a result of inadequate culture conditions, but although several attempts have been made to increase the yield of Type III structures by using different culture media (e.g. Weymouth’s medium, RPM-1640, and increased levels of essential amino acids) these have not met with any success.

A second striking feature is the presence in the most highly organized cultured ICMs of cells which resemble the extra-embryonic ectoderm and chorion of the normal 7-0–7-5-day p.c. C3H/He embryo (Fig. 6A, B). In the absence of specific intracellular biochemical markers, our tentative identification of these cells is based entirely on their morphology and tissue organization, and on the fact that cells resembling secondary trophoblast giant cells
are seen when mechanically disrupted Type III structures grow out on the surface of the culture dish (Fig. 7). The extra-embryonic ectoderm-like cells seem to develop from elongated cells which first make their appearance on about day 3 of culture as part of the inner vesicle of Type II structures.

There is evidence from embryo reconstruction experiments, in which ICMs isolated microsurgically from fully expanded 3-5-day p.c. mouse blastocysts are injected into trophoblast vesicles or blastocysts of a different genotype (Gardner, Papaionnou & Barton, 1973; Gardner & Papaionnou, 1975; Gardner & Johnson, 1975), that the extra-embryonic ectoderm and chorion is normally derived from polar trophoblast and not from the inner cell mass. In the light of these embryo reconstitution experiments there are four possible explanations for our results:

(1) In 25% of the blastocysts the immunosurgery procedure failed to kill all of the outer polar trophectoderm cells, so that one or two of them (possibly with lower antigen concentration on the surface) remained with the inner cell mass, and later, as a result of cell division, manifested themselves as extra-embryonic ectoderm. While we cannot totally eliminate this possibility we feel that it is very unlikely, for several reasons. Firstly, the embryos were incubated in a concentration of antiserum seven times higher than the minimum concentration found to be effective in the immunosurgery procedure (see Methods). Secondly, the concentration of complement used in these experiments was at least ten times higher than the minimum required to lyse all the outer cells previously exposed to a 1:30 dilution of antiserum. Finally, in a batch of fourteen blastocysts fixed 30 min after exposure to complement and serially sectioned at 1 μm, all of the outer trophectoderm cells were clearly dead, including those above the inner cell mass. (See also fig. 2 in Hogan & Tilly, 1978). Using other criteria, Handyside & Barton (1977) have also concluded that immunosurgery effectively kills all of the outer trophectoderm cells.

(2) At the stage when immunosurgery was performed, about 25% of the blastocysts may have begun to form ectoplacental cones, so that the polar trophoderm layer would be more than one cell thick above the inner cell mass. Thus, even if all of the outer trophectoderm cells are killed, one or two cells could remain with the inner cell mass and eventually give rise to extra-embryonic ectoderm. Nevertheless, in all blastocysts which were sectioned and examined in the electron microscope the polar trophoderm appeared to form a distinct monolayer over the inner cell mass (as noted also by Nadijcka & Hillman (1974) for substage four blastocysts). However, the morphology of the two cell types is very similar and we cannot exclude the possibility that internalization of some polar trophectoderm had taken place before immunosurgery.

(3) The mature chorion of the normal embryo may be of dual origin, containing extra-embryonic ectoderm cells derived both from the polar trophectoderm (ectoplacental cone) and from the inner cell mass. The embryo reconstruction
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experiments do not allow sufficient resolution to completely eliminate this possibility.

(4) Finally, some cells in the inner cell mass may remain pluripotent and able to give rise to trophoblast derivatives (extra-embryonic ectoderm) when faced with an abnormal environment in which no polar trophectoderm is present. This hypothesis is not incompatible with the results of the embryo reconstruction experiments described above since in those experiments donor trophectoderm tissue was always present.

At present, we consider either of the last two alternatives the most likely, but cannot distinguish between them. One way of eliminating hypothesis (2), however, is to study the in vitro development of inner cell masses isolated immuno- surgically from blastocysts around 3-5 days p.c., when it is very unlikely that internalization of the polar trophoblast would have occurred. The results of these experiments and a full discussion of the combined results are given in the following paper (Hogan & Tilly, 1978).

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REFERENCES


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