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Properties of Mouse Inner Cell Masses Isolated by Immunosurgery, Exposure to the Calcium Ionophore A23187 or Low Osmolarity

G. M. Harlow and P. Quinn

Department of Biological Sciences, University of Newcastle, Newcastle, N.S.W. 2308.

Abstract

Mouse inner cell masses remained intact when exposed to extreme osmotic stress (distilled water) for short periods, but the trophectoderm was lysed in one-third of blastocysts. However, these inner cell masses were not viable as they could not fluoresce after incubation in fluorescein diacetate nor continue development *in vitro*. It was concluded that cells of the inner cell mass are not more tolerant of osmotic stresses than those of the trophectoderm.

Inner cell masses were isolated from only 50% of blastocysts when exposed to the calcium ionophore A23187. Some lysing trophectoderm cells are probably able to restore their normal calcium levels after transfer to fresh culture medium allowing normal blastocyst-like development *in vitro*. Further evidence which suggests that not all trophectoderm cells are removed after incubation in ionophore is that these inner cell masses produced more trophoblast-like outgrowths *in vitro* and were able to induce the decidual cell reaction *in vivo* more often than their immunosurgically isolated counterparts.

Immunosurgically isolated inner cell masses were viable as they fluoresced brightly after incubation in fluorescein diacetate and developed normally *in vitro*. There was little or no contamination of these inner cell masses with trophectoderm cells as they formed considerably smaller outgrowths than control blastocysts and were rarely able to induce the decidual cell reaction *in vivo*.

Trophoblast-like giant cells were found less frequently from inner cell masses isolated from 142-h, post-HCG blastocysts and cultured *in vitro*, than from 118-h, post-HCG blastocysts. These results are discussed in relation to a current theory on the time of inner cell mass determination which states that apparently differentiated inner cell mass cells may reverse their direction of development in response to altered environmental conditions.

Introduction

The development of techniques for separating the inner cell mass (ICM) and trophectoderm of the mouse blastocyst has led to many studies examining the time of determination and subsequent fate of these two cell types. Three methods have been used for isolating ICMs—microsurgery (Gardner 1971; Gardner and Johnson 1972), immunosurgery (Solter and Knowles 1975; Harlow and Quinn 1979) and exposure to the calcium ionophore A23187 (Surani *et al.* 1978; Harlow and Quinn 1979).

Inner cell masses re-exposed to immunosurgery or ionophore are less susceptible to lysis than the trophectoderm (Harlow and Quinn 1979). Since these techniques rely on altering the ionic balance of the cell, there may be inherent differences between the properties of the ICM and trophectoderm such that the ICM cells are more tolerant of osmotic stresses. It was decided, therefore, to expose blastocysts to a short but extreme osmotic stress (distilled water) to examine whether ICM cells are better able to survive than trophectoderm cells.

Results from studies on the development of ICMs have appeared conflicting. Early work by Rossant (1975*a*, 1975*b*, 1976) using microsurgically isolated ICMs transferred back to a living host, showed that ICM cells did not contribute to trophectoderm derivatives. When immunosurgically isolated ICMs or dissociated cells from microsurgically isolated ICMs are injected into host blastocysts which are transferred to the uterus, the ICM cells also contribute only to the foetus and extra-embryonic membranes (Handyside and Barton 1977; Gardner and Rossant 1979; Rossant and Lis 1979*a*). Inner cell masses exposed to an external environment (i.e. left singly, fused with morulae or fused with mural trophectoderm) before transfer to the uterus, occasionally contribute to the trophoblastic as well as ICM derivatives of the embryo (Gardner *et al.* 1973; Rossant and Lis 1979*a*, 1979*b*). Culturing ICMs *in vitro* has also yielded varying results. In some cases, embryoid bodies form with no evidence of trophoblast outgrowth (Solter and Knowles 1975; Wiley *et al.* 1978; Hogan and Tilly 1978*a*; Surani *et al.* 1978). In other cases, trophoblast giant cells have developed from ICMs (Atienza-Samols and Sherman 1978), from clumps of dissociated ICM cells (Hogan and Tilly 1978*a*, 1978*b*) and from ICM 'cores' (Wiley 1978; Atienza-Samols and Sherman 1979). These studies suggest that some or all of the ICM cells are capable of altering their development in response to environmental changes till quite late in development. Since the conflicting results may be attributable to differences in technique with regard to the preparation of the ICMs or their age at isolation, we have investigated the development *in vitro* of ICMs isolated by immunosurgery, exposure to calcium ionophore or low osmolarity from different aged blastocysts.

Inner cell masses isolated microsurgically are not able to induce the decidual cell response (DCR) in the uterus (Gardner 1971; Gardner and Johnson 1972). However, aggregates of ICMs isolated immunosurgically from early blastocysts were able to induce a decidual reaction (Rossant and Lis 1979*b*). Therefore, the ability of ICMs isolated by immunosurgery or exposure to ionophore to induce the DCR in the uterus was also examined in the present study.

Materials and Methods

Embryos

Fully expanded zona-free blastocysts were obtained as previously described (Harlow and Quinn 1979). Briefly, embryos were collected between 1000 and 1200 h on the fourth day of pregnancy (day of plug = day 1) from superovulated, 3–5-week-old virgin mice of the F_1 hybrid cross (C57BL × CBA, or reciprocal cross). Most of the collected embryos were at the blastocyst stage and they were cultured in microdrops of Whitten's Medium (WM_1) under paraffin oil (Fisher Scientific Company, New Jersey, U.S.A.) in plastic culture dishes (No. 301V; Sterilin, Middlesex, U.K.) or microtest plates (No. 3034; Falcon, Oxnard, California, U.S.A.) for 26–28 h or 50–52 h at 37°C in a humidified atmosphere of 5% CO_2:5% O_2:90% N_2. The ages of the blastocysts which developed were either 118 h (range 115–121 h) or 142 h (range 139–145 h) post-HCG. The zonae pellucidae were removed from these cultured blastocysts by incubation in 0·5% Pronase (B grade, Calbiochem, Carlingford, N.S.W.).

Inner Cell Mass Isolation

(1) *Immunosurgery.* Blastocysts were incubated in anti-mouse spleen antiserum (1:50) for 15–30 min, washed in WM_1, then transferred to guinea pig complement (1:10, Commonwealth Serum Laboratories, Parkville, Vic.) for 30–60 min as described by Harlow and Quinn (1979).

(2) *Calcium ionophore.* Blastocysts were incubated in 10^{-5} M calcium ionophore A23187 (Lilly Industries, West Ryde, N.S.W.) prepared in modified WM_1 without calcium and magnesium ions, for 30 min in the dark (Harlow and Quinn 1979).

(3) *Low osmolarity*. Blastocysts were exposed to 1 ml triple glass distilled water and 5–10 blastocysts were removed at intervals of 1 min up to a maximum of 7 min exposure.

Embryos were examined using a Leitz Diavert microscope with phase optics and the extent of lysis determined visually. The viability of ICMs was assessed using a fluorescein diacetate (FDA) test (Mohr and Trounson 1980). Inner cell masses were incubated in FDA (5 μg/ml WM_1) for 1 min at room temperature and washed for a minimum of 1 min before preparing whole mounts which were kept on ice. These preparations were examined with a Leitz Orthomat microscope using phase-contrast optics and ultraviolet light. The combination of filters for the ultraviolet light consisted of a KP500 interference filter in the vertical illuminator and a K510 barrier filter. The amount of fluorescence was estimated semi-quantitatively using a centre-field light sample by recording the time taken to trigger the camera (ASA 400) when the embryos were exposed to ultraviolet light only. Inner cell masses were considered viable when they fluoresced brightly and triggered the camera after 5–10 s, the time required for ICMs from control blastocysts.

Inner Cell Mass Development in vitro

Some ICMs were transferred singly to 10 μl of medium in microtest plates under paraffin oil and incubated at 37°C in an atmosphere of 5% CO_2 in air for 4 days. The medium used was Eagle's minimum essential medium (MEM) supplemented with 26·2 mM sodium bicarbonate, 10% heat-inactivated foetal calf serum, penicillin (75 μg/ml), streptomycin (100 μg/ml) and kanamycin (100 μg/ml).

The development of ICMs was assessed by scoring the number which were still alive and those which exhibited trophoblast-like outgrowth. The diameters of live ICMs were measured. The outgrowths were washed with phosphate-buffered saline, fixed with ethanol–acetic acid (3:1, v/v) for 30–60 min, washed with ethanol for 30–60 min, then stained with lacto-orcein (Sherman and Atienza 1975). The largest and smallest diameters of each outgrowth were measured, from which the outgrowth areas could be calculated. The number of outgrowing cells in each outgrowth was recorded and the largest and smallest diameters of the nucleus in each outgrowing cell were measured.

Implantation in vivo

Inner cell masses isolated from 118-h, post-HCG blastocysts by immunosurgery or exposure to ionophore were transferred to the uteri of day 3 pseudopregnant recipients which had mated with sterility-tested vasectomized males. The transfer operation was similar to that described by Mullen and Carter (1973). In all, 6–11 ICMs were transferred to one uterine horn and 5–9 control blastocysts were transferred to the other uterine horn. The horns used for each treatment were randomly alternated among the transfers.

Two days after transfer, the recipient females were injected intravenously with 0·2–0·3 ml of 1% (w/v) Pontamine Sky Blue (6BX, Gurr, London, U.K.). They were killed 15 min later, their uteri removed and the number of Pontamine Sky Blue areas was recorded. The uteri were fixed and cleared according to the method of Orsini (1962) and the Pontamine Sky Blue areas examined more closely to confirm the exact numbers. The percentages of transferred embryos eliciting the DCR were transformed to angles and analysed on a weighted uterine horn difference as described by Bowman and McLaren (1970).

Results

Isolation of Inner Cell Masses

The response of blastocysts to incubation in distilled water is shown in Fig. 1. Half of the blastocysts collapsed after 1 min, approximately 80% by 2 min, and all had collapsed after incubation for 5 min. Lysis of the trophectoderm layer was first seen after 2 min and by the end of 4 min half of the blastocysts showed lysis of some of their trophectoderm cells. In 33±5% ($n = 321$, 24 replicates) of blastocysts, the trophectoderm layer had lysed, but the ICM remained intact. The number of blastocysts completely lysed increased, until by 7 min all were completely lysed.

Within 3 min, 70% (11 out of 19) of immunosurgically isolated ICMs were totally lysed in distilled water. The other 30% of ICMs had more than half of their cells lysed. By 4 min, all ICMs were totally lysed.

The age of the blastocyst had no effect on the success of ICM isolation. Immunosurgery yielded ICMs from 96±4% ($n = 227$, 9 replicates) of blastocysts. However, after exposure to ionophore, ICMs were isolated from only 44±9% ($n = 209$, 8 replicates) of blastocysts which is lower than that originally reported by Harlow and Quinn (1979). Fresh stocks of ionophore were prepared but were found to yield no more ICMs than the original stocks which were up to 12 months old. The difference in these results appears to lie in the different methods of assessing the extent of lysis. Previously an ICM was scored when the outer trophectoderm cells were seen to be lysing using Trypan Blue as an indicator of cell death. However, when these 'ICMs' were placed in MEM and their development followed, it became obvious that many of them were collapsed blastocysts showing only some of the trophectoderm cells lysing. Therefore, half of those originally classified as an ICM had not shown complete lysis of the trophectoderm cells and many (75%) were able to re-expand overnight and continue normal development *in vitro*.

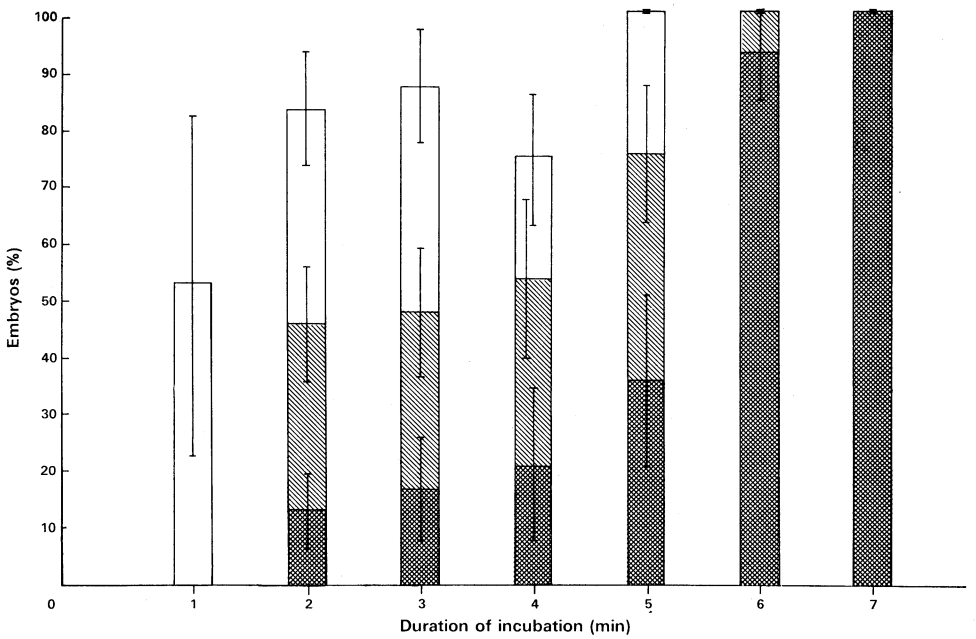

Fig. 1. Response of blastocysts to incubation in distilled water. Heights represent means with standard error bars based on 3–10 replicates with 5–10 blastocysts per replicate. Open histogram, collapsed blastocysts; oblique shading, collapsed blastocysts with trophectoderm cells lysed; double shading, collapsed blastocysts with both trophectoderm and ICM cells lysed.

Viability of Inner Cell Masses

When examined using the FDA test, immunosurgically isolated ICMs fluoresced brightly and took 9±1 s ($n = 22$) to trigger the camera. However, ICMs from water-shocked blastocysts showed only small patches of fluorescence, the intensity of which was significantly less than that of the fluorescence of immunosurgically isolated ICMs (time to trigger the camera = 16±2 s, $n = 13$; $t_{33} = 3{\cdot}62$, $P < 0{\cdot}002$).

The development *in vitro* of ICMs is given in Table 1. When cultured in MEM for 4 days, less than half of the ICMs were alive, as indicated by development in

culture. There were more ICMs alive when isolated by immunosurgery compared with the ionophore treatment although the difference was not significant. Only 8% of ICMs from water-shocked blastocysts were alive. The percentage of ICMs successfully developing *in vitro* decreased as the age of the blastocyst increased, but this also was not significant. The diameters of ICMs which did develop were similar for the 118- and 142-h, post-HCG blastocysts. Also shown in Table 1 are the percentage and diameters of ICMs which developed from control blastocysts. In this culture system, ICM growth is supported in only one-quarter of the blastocysts.

Table 1. Development of ICMs from blastocysts exposed to immunosurgery, calcium ionophore or distilled water, then cultured in MEM for 4 days
Values given are means ± standard errors

Age of blastocyst (h post-HCG)	Method of isolation	No. of replicates	ICMs alive (%) (total No. of blastocysts)	Diameter (μm) (No. of ICMs)
118	Immunosurgery	6	41±4 (51)	60±6 (15)
	Ionophore	4	33±12 (18)	80 (1)
	Water	3	8±8 (37)	—
142	Immunosurgery	4	31±15 (27)	65±6 (7)
	Ionophore	3	15±8 (13)	86 (1)
118	Control blastocysts	9	26±5 (401)	70±4 (9)

Trophoblast Regeneration from Inner Cell Masses

The extent of trophoblast outgrowth from ICMs and control blastocysts is given in Table 2. Few of the ICMs isolated after incubation in distilled water showed

Table 2. Development of trophoblast outgrowths from ICMs isolated by immunosurgery, exposure to calcium ionophore or distilled water and cultured in MEM for 4 days
Values given are means ± standard errors; total number of embryos used given in parantheses

Age of blastocyst (h post-HCG)	Method of isolation	No. of replicates	No. of outgrowths (%)	$10^{-3}\times$ Area (mm^2)	No. of cells
118	Immunosurgery	6	68±9 (51)	25±17 (17)	9±1 (17)
	Ionophore	4	79±8 (18)	45±17 (10)	13±3 (10)
	Water	3	11±11 (37)	41±10 (7)	15±1 (7)
142	Immunosurgery	4	43±18 (27)	31±11 (8)	16±3 (8)
	Ionophore	3	52±16 (13)	28±9 (3)	14±3 (3)
118	Control blastocysts	9	86±2 (401)	82±4 (164)	25±1 (146)

outgrowing trophoblast cells. More ICMs had outgrowing cells when isolated by ionophore rather than immunosurgery. Outgrowing cells were found in 75% of ICMs isolated from 118-h blastocysts, but in only 50% of ICMs isolated from 142-h blastocysts. There was little difference in the area or cell number of outgrowths

obtained from the two differently aged ICMs. Inner cell masses formed fewer outgrowths, which were less than half the area and contained half the number of cells as control blastocysts.

Nuclei diameters greater than 16 μm are assumed to be polyploid since a preliminary investigation showed that the diameters of nuclei from kidney, liver and day 14 embryo cells are all less than 16 μm. In all, 42% ($n = 266$ from 7 outgrowths) of nuclei in outgrowing cells from control blastocysts were less than 16 μm. The diameters of the nuclei in the outgrowing cells from ICMs were also measured: 67% ($n = 274$ from 27 outgrowths) of nuclei in outgrowing cells from 118-h ICMs were less than 16 μm in diameter, while 49% ($n = 116$ from 11 outgrowths) of nuclei in outgrowing cells from 142-h ICMs were less than 16 μm.

Implantation in vivo

Overall, $53 \pm 7\%$ (63 blastocysts transferred to 9 mice) of blastocysts were able to induce the DCR while only $24 \pm 11\%$ (79 ICMs transferred to 9 mice) of ICMs were able to do so. This difference is significant ($t_8 = 15{\cdot}31$, $P < 0{\cdot}001$) when analysed on a weighted uterine horn difference as described in the Materials and Methods section. Inner cell masses isolated by immunosurgery or exposure to ionophore behaved differently. Only 15% (8 out of 53) of immunosurgically isolated ICMs could induce the DCR, while 42% (11 out of 26) of ICMs isolated by exposure to ionophore could do so. More specifically, immunosurgically isolated ICMs failed to induce the DCR in four of six mice. In the remaining two mice, 2 out of 10 and 6 out of 11 ICMs could induce the DCR. Inner cell masses isolated by exposure to ionophore failed to elicit the DCR in one of three mice in which only two of five control blastocysts induced the DCR. In the other two mice, there was little difference between the number of DCR sites in horns receiving either control blastocysts or ICMs.

Discussion

It has been found that ICMs may be isolated from blastocysts by various techniques. A short extreme osmotic stress lysed the trophectoderm cells leaving the ICM intact in one-third of blastocysts. However, ICMs which had been isolated by immunosurgery, lysed just as quickly as trophectoderm cells when exposed to the distilled water. It does not appear, then, that blastomeres of the ICM are more tolerant of osmotic stresses than those of the trophectoderm. During brief periods of osmotic stress the ICM is protected until the trophectoderm cells lyse. Therefore, ICMs can be isolated if they are removed after the trophectoderm cells lyse but before they have been exposed to distilled water themselves. However, ICMs obtained this way are not viable as they do not fluoresce after incubation in FDA and very few are able to develop *in vitro*.

Exposing blastocysts to ionophore yields ICMs, but in many cases they are still contaminated with some intact trophectoderm cells. This was shown in three ways. Firstly, pure ICMs were obtained from only half of the blastocysts exposed to ionophore while ICMs were isolated from all immunosurgically treated blastocysts. Contaminating trophoblast cells were assumed to be present when the 'ICMs' were able to re-expand overnight and continue normal blastocyst-like development. Although the processes of cell lysis induced by immunosurgery and exposure to

ionophore are similar (Harlow and Quinn 1979), the initial alteration in ionic balance is brought about by entirely different means. When immunosurgically isolated ICMs are transferred back to fresh culture medium, any cells which have not completely lysed but in which the complement-dependent antibody cytotoxicity has begun, will die as the former process is not reversible. However, cells which have not completely lysed after exposure to ionophore may be able to restore their normal calcium levels when transferred to fresh culture medium and subsequently continue normal development. Two-cell and eight-cell mouse embryos are also able to continue normal development after a short incubation (less than 3 h) in medium devoid of calcium (Wales 1970; Ducibella and Anderson 1975).

The second evidence for trophectoderm cell contamination comes from the greater number and larger outgrowths from ionophore rather than immunosurgically treated 118-h blastocysts. Since the size of the outgrowth is directly proportional to the number of cells initially present (McLaren and Hensleigh 1975), ICMs isolated by ionophore have more cells capable of attaching and growing out along the dish than immunosurgically isolated ICMs. These extra cells are thought to be trophectoderm cells.

Thirdly, ICMs isolated by ionophore induced the DCR three times more often than those isolated by immunosurgery. Since single ICMs from fully expanded blastocysts are not able to induce the DCR in the uterus (Gardner and Johnson 1972), it is inferred that the majority of those ICMs capable of inducing the DCR were contaminated with some trophectoderm cells. It is possible that some decidualization observed after transfer of ICMs may have occurred due to the trauma of the uterine injection, though this is unlikely since in no case did all the transferred control blastocysts give rise to decidual swellings. Alternatively, the small incidence of decidual cell reaction induced by immunosurgically isolated ICMs may have arisen if these ICMs are not fully determined and are able to regenerate trophectoderm cells in response to an external environment. Although Rossant and Lis (1979*b*) found that aggregates of ICMs could induce the DCR, this situation may be different to that in single ICMs. Preliminary work in this laboratory has shown that when two or more ICMs are present in each well of microtest plate containing 10 μl of medium, they often form blastocyst-like vesicles irrespective of whether they have fused and hence may have different properties to those of a single isolated ICM.

Immunosurgery is the most efficient technique for obtaining a viable pure population of ICMs. Additional evidence of the purity of ICMs to that given by Handyside and Barton (1977) has been provided. These ICMs are viable since they fluoresce after incubation in FDA as well as ICMs in intact blastocysts. Also, the proportion of ICMs alive after 4 days of culture is not less than the number of ICMs which develop from control blastocysts in our culture system. Although outgrowing trophoblast cells consistently develop from control blastocysts after 4 days of culture, the development of the ICM is variable, with only 26% developing in the present study. Improved development of the ICM has since been achieved using Dulbecco's modified Eagle's medium. McLaren and Hensleigh (1975) have suggested that the source and batch of foetal calf serum also introduces variability.

There was a trend for ICMs isolated from the younger blastocysts to develop more outgrowing cells than those from the older blastocysts. This is consistent with the increasing number of reports which describe the formation of trophoblast-like giant cells from ICMs isolated at various times and cultured in different conditions

(Atienza-Samols and Sherman 1978, 1979; Hogan and Tilly 1978*a*, 1978*b*; Wiley 1978). Together, these results support the hypothesis put forward by Johnson *et al.* (1977) that irreversible determination of the ICM blastomeres does not occur till late in development and may actually occur after differentiation of the trophectoderm blastomeres. In response to an altered environment (e.g. culture *in vitro*) some apparently differentiated ICM cells may reverse their direction of development or 'de-differentiate' to form trophoblast-like cells which are able to attach and grow out along the culture dish.

The difference in these results to those of earlier reports claiming that ICM cells do not form trophectoderm derivatives (Rossant 1975*a*, 1975*b*, 1976; Gardner and Rossant 1979) is most likely due to two factors. Firstly, in the earlier work, ICMs were isolated by microsurgery which is performed on fully expanded blastocysts rather than by immunosurgery which can also be used for early blastocysts. Irreversible determination of the ICM blastomeres may have already occurred in the fully expanded blastocysts. Secondly, the development of the ICM cells was earlier followed *in vivo* rather than *in vitro* where the culture environment may influence cells to develop in a different way.

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