Evaluation of the technique of immunosurgery for the isolation of inner cell masses from mouse blastocysts

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SUMMARY

Inner cell masses (ICMs) immunosurgically-isolated from 3½-day mouse blastocysts were examined for trophoblast cell contamination and developmental capacity. Blastocysts were preincubated in rabbit anti-mouse antiserum, washed thoroughly and then incubated in complement. The ICMs were then easily dissected by drawing through a fine pipette.

Various experiments confirmed that the trophectoderm had been completely removed by this treatment. Firstly, the ICMs did not bind a fluorescein-conjugated antibody directed against rabbit IgG, indicating the absence of cells exposed to the rabbit antiserum during the immunosurgical procedure. Secondly, ICMs dissected from blastocysts preincubated in a suspension of melanin granules did not include any of the trophoblast cells that had phagocytosed the granules. And, thirdly, the protein synthetic profile of these ICMs was similar to microsurgically dissected ICMs, and in particular, trophoblast specific spots were absent.

The developmental capacity of immunosurgically-isolated ICMs was tested by injecting them into blastocysts and transferring to the uterus of 2½-day pseudopregnant recipients. Extensive chimaerism was detected in the majority of implants, 5–6 days after transfer, but only in ICM-derived tissues. This demonstrates both the lack of trophoblast cell contamination and functional viability of these ICMs.

INTRODUCTION

The 3½-day mouse blastocyst consists of two distinct groups of cells: the inner cell mass (ICM) and the trophectoderm. The properties of these tissues have been extensively studied after their microsurgical separation (Gardner, Papaioannou & Barton, 1973; Rossant, 1975a, b; Van Blerkom, Barton & Johnson, 1976). However, the difficulty of the microsurgical procedure limits the rate at which blastocysts can be dissected. Furthermore, some ICM cells may become damaged due to the use of needles to pull the ICM away from the overlying trophectoderm. An alternative technique for the isolation of ICMs, termed immunosurgery, has recently been described (Solter & Knowles, 1975). Blastocysts were preincubated in anti-species antiserum, washed thoroughly and then incubated in complement. Since the antiserum was prevented from gaining access to the ICM by the tight junctions between the surrounding

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trophectoderm cells, only trophoblast tissue lysed on the addition of complement, leaving the ICMs intact. This technique has the potential advantage of allowing many ICMs to be recovered without the risk of mechanical damage to any of the cells. In the experiments of Solter & Knowles, the purity of recovered ICMs was assessed by the failure to detect trophoblastic-type outgrowths in vitro. We report here a more systematic assessment of the purity of immunosurgically-derived ICMs, as well as a test of their developmental capacity.

The first three experiments reported here were carried out to determine whether immunosurgically isolated ICMs were contaminated with any trophoblast cells resistant to complement lysis. First, a fluorescent-conjugated anti-Ig antiserum was used to detect antibody bound to the surface of any remaining trophoblast cells after immunosurgery. Secondly, blastocysts were incubated in suspensions of melanin granules prior to immunosurgery, so that the trophoblast cells, which selectively phagocytose the granules (Gardner, 1975), could be identified by phase contrast and electron microscopy at various stages in the immunosurgical procedure. Thirdly, the protein synthetic profile of immunosurgically-isolated ICMs was compared with the profiles of microsurgically isolated ICMs and trophoblast vesicles (Van Blerkom et al. 1976).

In a fourth experiment, the viability and developmental potential of immunosurgically-isolated ICMs was studied to determine whether exposure to complement or any other factors had affected them adversely. The ICMs were injected into genetically distinct host blastocysts and then transferred to pseudopregnant recipients. The contribution of the donor ICMs to post-implantation tissues was assessed by use of glucose-phosphate isomerase (GPI) isoenzyme markers several days later (Gardner et al. 1973).

MATERIALS AND METHODS

Immunosurgery

Blastocysts were flushed from the uteri of superovulated females early on the fourth day after mating. The zonae were removed by incubating in Pronase (Calbiochem 0·5 % Tris-citrate buffer pH 7·0) for 5–10 min at 37 °C, or by minimal (15–30 sec) exposure to acidic Tyrode’s solution, pH 2·5, adjusted with N/5 HCl, containing 0·4 % polyvinylpyrrolidone followed by transfer to medium PBl+10 % Foetal Calf Serum (FCS) (Whittingham & Wales, 1969), which was used thereafter for handling the embryos. Blastocysts treated with acidic Tyrode to remove their zonae implanted and developed normally after transfer to pseudopregnant recipients.

Blastocysts were then preincubated in a rabbit antiserum to mouse embryo homogenate (Gardner & Johnson, 1973) diluted 1 in 10 with PBl for 15 mins at 37 °C followed by three 5-min washes in PBl+10 % FCS. They were then transferred to guinea-pig complement (Flow Labs.) made up to the specified volume with the diluent supplied and then diluted 1 in 10 with PBl, and in-
cubated for up to 45 mins at 37 °C. These conditions had been shown in preliminary experiments to give optimal lysis with this antiserum and complement. The ICMs were dissected free of lysed trophoblast cells by drawing the embryos through a fine pipette and were then transferred to fresh medium.

**Immunofluorescence**

Control blastocysts, blastocysts after incubation with the rabbit antiserum and immunosurgically-isolated ICMs were incubated under oil in separate wells of a cytotoxicity slide for 15 min at 4 °C in fluorescein-conjugated goat anti- rabbit-IgG (Miles Labs.) diluted 1 in 10 with PBl (Muggleton-Harris & Johnson, 1975). The embryos were then transferred with minimal amounts of medium through three more wells containing drops of PBl + 10 % FCS to wash off excess conjugate before removing the slide from the oil, applying a coverslip and viewing with a Zeiss epifluorescent microscope (incident fluorescent light source HBO 200 with excitation filter system 427902 and barrier filter system 427903).

**Incorporation of melanin granules**

Blastocysts were incubated for 2 h in a suspension of melanin granules in PBl + 10 % at 37 °C before immunosurgery. The suspension was prepared by scraping fragments of melanin from the pigmented layer of the eye of C57Bl mice in a little medium, and decanting to eliminate large fragments (Gardner, 1975).

**Protein synthetic analysis**

Groups of 8–24 control blastocysts and of immunosurgically-isolated ICMs were incubated for 2–4 h in protein-free medium 16 (Whittingham, 1971) in the presence of [35S]methionine. The embryos were then lysed and the labelled protein analysed by two-dimensional electrophoresis and fluorography as described elsewhere (O'Farrell, 1975; Van Blerkom et al. 1976).

**Injection of ICMs into blastocysts**

ICMs were immunosurgically dissected from blastocysts derived from matings homozygous for GPI-1b and injected into host blastocysts homozygous for GPI-1a, using of a Leitz micromanipulator. The injection chimaeras were then transferred to 2½-day pseudopregnant CFLP recipients homozygous for GPI-1a. Six or seven days after transfer the conceptuses were explanted and dissected into ectoplacental cone, mural trophoblast (including Reichert’s membrane), yolk-sac, amnion and allantois (analysed together) and conceptus. Each of these five samples of tissue was then washed separately in drops of medium, and lysed by freezing and thawing in 5 μl of distilled water. The lysates were then run on starch slab gels at 4 °C, 170 V for 45–60 min to determine the GPI isoenzyme variants present. Samples of diluted blood from GPI-typed animals were run as controls (Gardner et al. 1973).
Phase contrast micrographs of 3½-day blastocyst after incubation in a suspension of melanin granules (A) prior to lysis, granules present in the trophoblast cells (× 400); (B) undergoing complement lysis after antibody treatment (× 400) and (C) isolated ICMs post-lysis aggregating in culture and showing no melanin granules (× 400).
**Electron microscopy**

Control blastocysts, blastocysts after incubation with melanin granules, and ICMs isolated immunosurgically from blastocysts that had been incubated with granules were prefixed for 30 min at 4 °C in Karnovsky’s fixative (Karnovsky, 1965), washed twice in 0.1 M sodium cacodylate and then postfixed in 1 % osmium tetroxide for 45 min at 4 °C. They were then dehydrated in graded concentrations of acetone before embedding in araldite. Sections, 5 nm thick, were stained with lead citrate and uranyl acetate. Unstained sections were viewed at 60 kV. Sections were viewed on the Philips 300 electron microscope.

**RESULTS**

**Immunofluorescence**

After incubation in rabbit antiserum both polar and mural trophoblast showed an intense and specific uniform surface staining. However immunosurgically-isolated ICMs did not show any fluorescence, thus indicating that none of the outer trophoblast cells remained present on the ICM after treatment with complement.

**Melanin granules**

Blastocysts before dissection showed presence of granules both in mural and polar trophoblast under phase contrast and by electron microscopy (Figs. 1A, 2A). After immunosurgery cells bearing granules were absent (Figs. 1C, 2B). Furthermore, isolated ICMs aggregated spontaneously in culture (Fig. 1C), a property not manifested by trophoblast cells or intact blastocysts.

**Protein synthetic patterns**

The patterns obtained with immunosurgically-isolated ICMs were similar to those obtained with microsurgically-isolated ICMs of equivalent age. In particular all trophoblast specific spots were absent but those characteristic of the ICM were present (Fig. 3). The absolute incorporation of label was greater than that obtained with microsurgically-isolated ICMs.

**ICM injection into blastocysts**

Of 18 injection chimaeras, 16 transferred blastocysts subsequently implanted. The ICM-derived tissues (amnion, allantois, yolk-sac, embryo) of 14 out of these 16 showed contributions of donor ICM GPI-type. Two conceptuses also showed some contribution of the donor ICM tissue to trophoblast-derived tissues (Fig. 4). This was probably due to contamination of mural trophoblast with distal endoderm and of ectoplacental cone by adherent allantois during dissection (Gardner et al. 1973).
FIGURE 2
Electron micrograph of (A) mural trophoblast showing granules both on the surface and within the cytoplasm, and a tight junction between two trophoblast cells ($\times 15000$); (B) ICM immunosurgically-isolated from blastocyst incubated in melanin granules, with no adherent or contained granules ($\times 3900$).
Two-dimensional electrophoresis patterns of radioactive proteins synthesized by (A) microsurgically-isolated trophoblast; (B) immuno-surgically-isolated ICMs; (C) microsurgically-isolated ICMs. (Arrowheads indicate trophoblast characteristic spots, and arrows those characteristic of ICM.) Separation horizontally by isoelectric focusing and vertically by SDS gradient electrophoresis.
DISCUSSION

Trophoblast cells could not be detected after immunosurgical isolation of ICMs from 3½-day mouse blastocysts, as judged by several independent criteria.

Firstly, no cells could be detected by immunofluorescent staining of bound antibody or by presence of melanin granules despite the fact that both these markers were present on or in all trophoblast cells before immunosurgery. Secondly, the patterns of protein synthesis of the ICMs showed none of the spots specific for microsurgically-isolated trophectoderm but did show ICM specific spots (Van Blerkom et al. 1976). And thirdly, chimaeras formed by injection of immunosurgically-isolated ICMs into host blastocysts showed donor ICM contribution to trophoblast-derived tissues in only two cases. A contaminant allantois was almost certainly responsible for the contribution to the ectoplacental tissue of the 9½-day embryo since this organ was missing from the embryonic dissection. The trace of activity found in mural trophoblast and associated Reichert's membrane of these older embryos was probably due to distal endoderm.

Additional evidence on the purity of immunosurgically-isolated ICMs has come from the analysis of three interspecific chimaeras made by injecting immunosurgically-isolated rat ICMs into mouse blastocysts (M. H. Johnson and R. L. Gardner, personal communication). The rat ICMs contributed only to embryonic ectoderm and to extra-embryonic and embryonic endoderm, but not to trophoblast, ectoplacenta and extra-embryonic ectoderm, indicating possession of the same developmental potential as microsurgically-isolated ICMs (Gardner & Johnson, 1975).

These experiments have also reduced doubts that the ICMs are deleteriously
affected by the process of immunosurgery. The high proportion of chimaeric embryos recovered and the generally high contribution to embryonic tissues indicate that the functional viability of these ICMs is at least as good as their microsurgically-dissected counterparts. Indeed the increased incorporation of $[^{35}\text{S}]$methionine into protein by immunosurgically-isolated ICMs as compared with microsurgically-isolated ICMs may be a reflexion of the better condition of the cells.

Immunosurgery allows only the isolation of ICMs and although an alternative technique has been described for the isolation of trophoblast vesicles, reservations have recently been expressed about the use of $[^3\text{H}]$thymidine in this approach (Rossant & Kelly, 1976). Microsurgical methods may still be the only way of recovering this component of the blastocyst. The real advantages of immunosurgery are the ease with which larger numbers of blastocysts can be dissected and the possibility of obtaining ICMs from strains which are difficult to handle microsurgically.

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REFERENCES


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