Interaction between inner cell mass and trophectoderm of the mouse blastocyst
I. A study of cellular proliferation

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SUMMARY

Increase in cell number has been compared with mitotic activity in the polar and mural trophectoderm and in the inner cell mass (ICM) of mouse blastocysts. The results indicate that whereas ICM cells divide at a rate which is compatible with the rate of increase of ICM cell number, polar trophectoderm cells divide faster and mural trophectoderm cells slower than can account for their own rates of cell number increase. It is suggested that the ICM induces a high rate of proliferation in the polar trophectoderm and that there is a resulting cell shift from polar to mural regions during blastocyst development. Mural trophectoderm cells close to the ICM divide faster than those farther away, indicating that cells may retain a 'memory' of ICM contact for some time after leaving the ICM. There is considerable cell death in the blastocyst, but this is restricted to a short period of time coincident with the appearance of primitive endoderm.

INTRODUCTION

Proliferation of mouse postimplantation trophoblast requires the presence of inner cell mass cells (Gardner, 1971). However, there is no cellular contribution from the inner cell mass (ICM) to ectoplacental cone or extra-embryonic ectoderm tissues (Gardner & Johnson, 1975; Gardner, Papaioannou & Barton, 1973) and so it has been suggested that the ICM interacts with overlying trophoblast to promote its proliferation. It is not known whether this interaction operates in the preimplantation mouse blastocyst, although by 4½ days post coitum (p.c.) the first signs of giant cell transformation are evident in the mural trophectoderm directly opposite the ICM (Dickson, 1963, 1966). Cells with DNA contents exceeding 4C first appear at this time (Barlow, Owen & Graham, 1972) and furthermore, trophectodermal proliferation after blastocyst attachment in culture requires the presence of ICM cells (Ansell & Snow, 1975). Thus it is possible that the ICM may effect differential rates of proliferation in the polar and mural trophectoderm of the preimplantation mouse blastocyst. An attempt has been made to investigate this possibility. The increase in cell number and distribution of mitotic activity have been analysed in the polar and mural trophectoderm and in the ICM of mouse blastocysts, from the time of their

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formation until implantation. Several studies have provided information on cell numbers and mitotic activity for the rodent blastocyst (Smithberg & Runner, 1960; Baevsky, 1963; McLaren, 1968; Barlow, Owen & Graham, 1972; McLaren & Bowman, 1973; Surani, 1975; Wu, 1976; Smith & McLaren, 1977; Handyside, 1978) but these have not covered the whole blastocyst period or attempted to distinguish between all the different regions of the embryo. Moreover, no account has been taken of the occurrence of cell death reported for the ICM of mouse blastocysts (Wilson & Smith, 1970; El Shershaby & Hinchliffe, 1974). In the present study the technique of reconstruction from serial histological sections has been used to obtain accurate counts of total cell numbers, mitoses and dead cells in mouse blastocysts.

After implantation mural trophectoderm cells cease dividing and undergo primary giant cell transformation (Alden, 1948). However, some cells in the ectoplacental cone remain diploid and mitotically active despite being separated from ICM derivatives by several trophoblast cell layers. Only peripheral ectoplacental cone cells transform into secondary giant cells (Duval, 1892; Snell & Stevens, 1966). Clearly ICM contact per se is not required for trophoblast proliferation, since cells which have lost contact with the ICM may continue dividing for some time. It is possible that the inductive stimulus is diffusible and acts over a distance, or alternatively, that cells retain the ability to divide for a certain length of time after losing contact with the ICM (Gardner & Papaioannou, 1975).

MATERIALS AND METHODS

Embryos

Embryos were obtained from random-bred CFLP mice (Anglia Laboratory Animals Ltd.) which were maintained under a regime of 12 h light and 12 h dark. Ovulation was assumed to occur at the mid-point of the dark period which was either at 1.00 a.m. or 7.00 p.m. Throughout this paper post-coital ages of embryos are estimated from the time of assumed ovulation. Oestrous females were placed with fertile males and mating was ascertained by presence of a copulation plug the following morning. At various times between 3 days 7 h p.c. and 4 days 20 h p.c. pregnant females were injected intraperitoneally with 60 μg colcemid (Ciba) (Tarkowski, 1966) dissolved in 0-1 ml phosphate buffered saline and were killed 2 h after injection. Control females were uninjected. Up to 4 days 11 h p.c. embryos were flushed from the uterine horns using PBI medium plus 10% heat-inactivated foetal calf serum (Whittingham & Wales, 1969). Blastocysts were recovered from the flushings and pipetted into the ampullae of oviducts which had been removed earlier from females on day 1 of pseudopregnancy. Each oviduct received embryos from a single pregnant female only, and was fixed immediately in formol-acetic-alcohol. This use of oviducts has been found to give consistently good histological preparations. From 4 days 12 h p.c. onwards it was difficult to flush blastocysts from the uteri.
due to the onset of implantation and so, in these cases, uteri were removed from females and fixed in formol-acetic-alcohol. Oviducts and uteri were dehydrated and embedded in paraffin wax (M.P. 56 °C) (Orsini, 1962), serially sectioned at 5 μm and stained with haemalum and eosin.

**Analysis**

Only expanded blastocysts found to be undamaged in all sections were used. Each section was drawn on tracing paper at ×1100 magnification using a Zeiss drawing tube attached to a Zeiss microscope. It was not always possible to see cell outlines, so the analyses were based on counts of nuclei which were drawn in outline and scored as being in interphase, mitotic or dead. Furthermore, nuclei were assigned either to the ICM (including visceral and parietal endoderm) or to the trophectoderm. Where the major part of a trophectodermal nucleus was found to lie over the ICM it was classified as polar, and where most of the nucleus lay over the blastocoel, as mural (see Fig. 1 A). In blastocysts in which endoderm had formed (Fig. 1 B) trophectodermal nuclei were considered to be polar only if there were at least two cell layers (including a single layer of visceral endoderm and one or more layers of primitive ectoderm) beneath them. Trophectodermal nuclei overlying a single cell layer (parietal endoderm) were classified as mural. In addition, those mural nuclei appearing farthest from the ICM were called distal (approximately one-quarter to one-third of the mural region was included in this category) and the remainder were said to be proximal. Since it was not possible to control the planes of section through blastocysts, oblique sections were often encountered and so the assignment of mural nuclei to proximal and distal groups was inevitably approximate. Interphase and mitotic nuclei were always clearly distinguishable. Prophase stages were included in the interphase category so that only metaphase, anaphase or, occasionally, telophase were classified as mitotic figures. A cell was considered dead according to the following criteria modified after Silver & Hughes (1974): (a) the nucleus was not less than approximately one-quarter the size of a healthy interphase nucleus, (b) was heavily eosinophilic and contained one or more rounded, regularly shaped chromatin masses, (c) the nucleus was contained within the blastocyst boundaries (excluding the blastocoel) and (d) was separated from all other putative dead cells by at least one healthy cell. These criteria aimed to distinguish dying cells from digestive vacuoles on the basis of size (criterion (a)) and from healthy cells on the basis of morphology (criterion (b)). Furthermore, criterion (c) excluded cells of possible extra-embryonic origin and also dying blastocyst cells whose region of origin was in doubt. Finally, criterion (d) made allowance for fragmentation during cell death so that several adjacent ‘dying cells’ were counted as a single dead cell. It is likely that these criteria resulted in an underestimation of the actual extent of cell death since, for example, in some cases dead cells were seen in the blastocoel and so discounted. On the other hand, an overestimate of the extent of cell death could
have been obtained if dying cells persist for a long time. In other developing systems the clearance of necrotic debris may take anything from hours to days to be completed (Glucksmann, 1951), and it is difficult to predict the length of time required in the mouse blastocyst. This uncertainty associated with the temporal aspects of cell death made any quantitative treatment, other than the calculation of dead cell indices, impractical.

Mitotic indices and dead cell indices were found separately for each blastocyst region, and for each classification group of blastocysts (classified according to age \( p.c. \) or to blastocyst total cell number). For example the mitotic index in the ICM of blastocysts containing a total of 40 to 49 cells was found by the formula:

\[
\frac{\text{total number of ICM mitoses in blastocysts containing 40–49 cells}}{\text{total number of ICM cells in blastocysts containing 40–49 cells}} \times 100.
\]

Dead cell indices were calculated in the same way.

**RESULTS**

The results are arranged in five sections. First it was necessary to establish whether colcemid-treated (CT) blastocysts differed from non colcemid-treated (NCT) control blastocysts with respect to cell numbers, mitoses and cell death. Second, results are presented on the relationship of blastocyst total cell number (BTCN) to estimated age \( post \ coitum (p.c.) \) Third, the blastocyst regions (mural and polar trophectoderm and ICM) are compared in terms of cell number, mitotic index and dead cell index in blastocysts of increasing total cell number. Fourthly, results on the distribution of mitoses within the mural trophectoderm...
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Table 1. Average ratios of ICM cell number to blastocyst total cell number for blastocysts of increasing size

<table>
<thead>
<tr>
<th>Blastocyst total cell number</th>
<th>Number of blastocysts</th>
<th>Number of litters*</th>
<th>Average</th>
<th>ICM cell number BTCN</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Analysed</td>
<td>Colcemid treated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30-39</td>
<td>10</td>
<td>10</td>
<td>7</td>
<td>0.41</td>
</tr>
<tr>
<td>40-49</td>
<td>3</td>
<td>2</td>
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<td>0.41</td>
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<tr>
<td>50-59</td>
<td>12</td>
<td>11</td>
<td>9</td>
<td>0.38</td>
</tr>
<tr>
<td>60-69</td>
<td>18</td>
<td>10</td>
<td>10</td>
<td>0.42</td>
</tr>
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<td>70-79</td>
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<td>5</td>
<td>0.37</td>
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<td>7</td>
<td>6</td>
<td>0.34</td>
</tr>
<tr>
<td>90-99</td>
<td>13</td>
<td>9</td>
<td>9</td>
<td>0.34</td>
</tr>
<tr>
<td>100-109</td>
<td>13</td>
<td>8</td>
<td>10</td>
<td>0.39</td>
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<td>110-119</td>
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<td>3</td>
<td>5</td>
<td>0.36</td>
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<tr>
<td>120-129</td>
<td>7</td>
<td>6</td>
<td>7</td>
<td>0.35</td>
</tr>
<tr>
<td>130-149</td>
<td>5</td>
<td>5</td>
<td>4</td>
<td>0.32</td>
</tr>
</tbody>
</table>

* Net total of litters used = 46.

are presented. Finally the number of ICM cells as a proportion of BTCN is given for blastocysts of increasing total cell number. A semi-quantitative analysis of cell number and cell division data is also presented (see Appendix). This tests whether, during blastocyst development, the observed cell number increase in each region is compatible with the rate of cell division in the same region.

Effects of colcemid treatment

Forty-six pregnant females yielded a total of 304 blastocysts, of which 102 were suitable for analysis according to criteria defined in Materials and Methods (Table 1). Blastocyst total cell numbers ranged from 30 to 140. Blastocysts containing 30-39 cells had probably recently cavitated since, in another random-bred strain, blastocoel formation has been found to occur between the 28- and 33-cell stages (Smith & McLaren, 1977). Blastocysts containing 140-149 cells showed contact between trophectoderm and uterine epithelium, indicating the onset of implantation. Thus it is considered that the present study spans the whole blastocyst period of development. Seventy-five of the 102 blastocysts analysed had been treated with colcemid for 2 h prior to fixation, and 27 were from untreated control females. Average total cell numbers and numbers of mitoses and dead cells were compared separately for the three regions of CT and NCT blastocysts using $\chi^2$ tests. Average total cell numbers did not differ significantly between CT and NCT blastocysts ($P > 0.05$) and nor did the rates of increase of regional cell numbers ($P > 0.05$). Furthermore, numbers of dead cells did not differ significantly between CT and NCT blastocysts ($P > 0.05$) but CT blastocysts had significantly more mitoses than NCT blastocysts in all regions ($P < 0.01$ for mural and ICM, $P < 0.001$ for polar). As a result of this
Fig. 2. The relationship of blastocyst total cell number (BTCN) to age of embryos post coitum (h). The regression line of best fit is included (see text).

analysis, CT and NCT data have been pooled for cell number and dead cell analyses, while mitotic index analyses are based solely on the CT group.

**Relationship between BTCN and estimated age p.c.**

Figure 2 shows the relationship of BTCN to age p.c. A regression analysis produced a best fit line of equation:

\[ y = 2.56x - 159.95 \]

and this is included in Fig. 2. There is clearly a strong association between BTCN and age p.c. since the regression coefficient differs significantly from zero \((P < 0.0001)\) and the rank correlation coefficient is similarly significant \((r = 0.81, P < 0.001)\). However, from Fig. 2 it can be seen that there is considerable variation in BTCN between embryos of the same age p.c. This may be due mainly to between-litter variation indicating a difference in mating times between females, but could also include within-litter variation. To resolve this problem an analysis of variance was performed using a randomized block factorial design. The power of such tests increases as the number of blocks (litters) and treatment levels (individual embryos within litters) increase (see Kirk, 1968 for discussion). In the present case it was necessary to select a particular age p.c. which gave the largest possible number of blocks and treatment levels in order to maximize the power of the test. Four litters each of four
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Embryos were randomly chosen from litters aged between 87 and 89 h p.c. and BTCN's compared. Both variation between litters \( (F = 8.79; \text{d.f.} = 3, 8; P < 0.01) \) and within litters \( (F = 17.12; \text{d.f.} = 3, 8; P < 0.001) \) was significant. Since such large variation exists between litters and between litter-mates it was felt that BTCN is a better measure of developmental stage than age p.c., and further comparisons were made, wherever possible, using BTCN as the index of developmental stage (Table 1).

**Analysis of regional cell numbers, mitotic indices and dead cell indices**

The data are presented in Fig. 3. Cell numbers (Fig. 3A) in the mural and ICM regions show approximately sevenfold and threefold increases respectively between blastocyst formation (BTCN = 30–39) and the onset of implantation (BTCN = 130–149), while the polar region only doubles its cell number during the same period and shows no marked increase in cell number at all up to the 100-cell stage. Figure 3B shows average mitotic indices for the three regions. Polar mitotic indices never fall below mural mitotic indices and at most blastocyst stages exceed mural values by a factor of two. Marked fluctuations in mitotic activity occur with peak values in blastocysts containing 30–59 and 70–89 cells. These fluctuations may reflect partial synchrony at the sixth (32–64) and seventh (64–128) cleavage divisions, occurring particularly in the ICM. Despite this possible division synchrony, mitotic activity in the ICM is approximately equal, on average, to that in the polar trophectoderm. In all regions mitotic indices fall in the latter half of the blastocyst period, and reach relatively low values in blastocysts with the highest cell numbers. Figure 3C shows regional dead cell indices. It appears that although dead cells occur in all three regions, cell death is rare in the mural trophectoderm. There is a well-defined peak of cell death in blastocysts containing from 60 to 110 cells, and up to 10% of ICM and polar cells are dead in blastocysts of the 70–89 cell group. This peak corresponds to a high level of mitotic activity, especially in the ICM, and to the start of primitive endoderm formation. Cell death falls to low levels near implantation.

From these results it would appear that the polar trophectoderm is a fast dividing region which does not markedly increase its cell number throughout blastocyst development whereas the mural region divides slowly and grows considerably. The rate of cell division in the ICM appears approximately compatible with its rate of cell number increase (see Appendix for further analysis).

**Distribution of mitoses within the mural trophectoderm**

All mural nuclei were classified as either proximal (near the ICM) or distal (farthest from the ICM, see Fig. 1). Despite the limitations of this method (see Materials and Methods) a comparison of mitotic indices for the mural sub-regions averaged over all 75 CT blastocysts would seem to be valid. The distal subregion comprised 25.5% of the mural region on average, and had a mitotic
Fig. 3. The relationship of blastocyst total cell number to (A) average cell number, (B) average mitotic index and (C) average dead cell index, for the mural (empty bars, dotted lines) and polar (hatched bars, dashed lines) trophectoderm and the ICM (filled bars, solid lines). Standard deviations are shown on the bar chart.
index of 2-0% (14 mitoses in 681 cells). The proximal subregion had a mitotic index of 7-1% (142 mitoses in 1992 cells). The difference is highly significant ($\chi^2 = 18-92$, d.f. = 1, $P < 0-001$). It is unlikely that proximity to parietal endoderm cells is the reason for the higher mitotic index in the proximal mural subregion since proximal mural mitotic indices significantly exceed distal mural mitotic indices both in blastocysts containing less than 100 cells, which are not expected to contain parietal endoderm ($\chi^2 = 7-94$, $P < 0-01$), and in blastocysts containing more than 100 cells in which parietal endoderm is forming ($\chi^2 = 15-94$, $P < 0-001$). In fact, in the latter group of blastocysts, no distal mitoses were seen at all.

**ICM cell number as a fraction of BTCN**

The ratio of ICM cell number to BTCN was found for each blastocyst separately and is expressed as an average value for each BTCN class in Table 1. The ratio falls throughout the blastocyst period, and the regression coefficient ($b = -0-0007$, $t = 3-50$, $P < 0-001$) and correlation coefficient ($r = 0-31$, $t = 3-34$, $P < 0-005$) are both significant.

**DISCUSSION**

**Blastocyst regional proliferation**

This paper presents data on the rates of proliferation of different regions within the mouse blastocyst. From Fig. 3 it can be seen that whereas ICM and polar trophectoderm cells divide at approximately equal rates, there is an imbalance in the rates of cell number increase in the two regions. Death of 'excess' polar cells is not a sufficient explanation since Fig. 3C shows that the extent of cell death in the two regions is approximately equal. Consequently, it may be predicted that viable cells leave the polar region as blastocysts develop. The mural trophectoderm divides rather slowly, but has a disproportionately high rate of cell number increase, possibly indicating a shift of polar cells into mural region. Selective polar labelling early in the blastocyst period has provided a direct demonstration of this cell movement during blastocyst development (A.J.C. in preparation). Recruitment of ICM cells into the mural region cannot at present be ruled out but is felt to be unlikely since there is considerable evidence that by 3½ days p.c. the ICM and trophectoderm of the mouse blastocyst belong to separate cell lineages (Rossant, 1975a, b, 1976). Furthermore, data presented in this paper indicate that the ICM has a rate of cell division which is compatible with its rate of cell number increase (see Appendix).

Two of the outstanding problems of early mouse development are as follows: First, why does trophoblast giant cell transformation begin directly opposite the ICM (Dickson, 1966)? Secondly, what mechanism is responsible for the origin of ectoplacental cone and extra-embryonic ectoderm at implantation.
while the polar trophectoderm remains as a single layer until this time? The results presented in this paper provide possible answers for both of these questions. The analysis of the distribution of mitoses within the mural region shows that proximal trophectoderm cells divide faster than distal cells. It is proposed that while ICM contact induces trophectodermal cell division, trophectoderm cells retain a 'memory' of this contact and proliferate for some time after leaving the ICM. Mural cells opposite the ICM are the earliest to lose contact with the ICM and so would be expected to cease dividing and form giant cells first. Subsequently, more proximal cells undergo the same transformation. It has been found that distal mural cells have ceased cell division in blastocysts containing more than 100 cells. Presumably these 'oldest' mural cells originated at blastocoel formation, approximately 24 h previously. An alternative mechanism could involve a gradient of a diffusible 'inducer', originating from the ICM and acting over a distance. However, this hypothesis does not explain why, after implantation, ectoplacental cone cells continue dividing although at a much greater distance from ICM derivatives than are trophoderm cells when they stop dividing in the blastocyst. On the other hand, a 'cell memory' mechanism overcomes this problem since it considers that dividing ectoplacental cone cells have been out of contact with ICM derivatives for a shorter time than non-dividing blastocyst trophectoderm cells, and that the greater distance of ectoplacental cone cells from ICM derivatives is due to cell movements occurring at this time.

Egg-cylinder formation may be explained by the following hypothesis. The polar region continues to proliferate throughout the blastocyst period, and continually gives rise to new proximal mural cells. However, at the time of blastocyst attachment to the uterine epithelium mural cells become lodged in the endometrium. Furthermore, the blastocyst is clamped within the uterine luminal crypt, and so any further influx of polar cells into the mural region of the implanting blastocyst is prevented. Consequently, continued polar proliferation produces an accumulation of trophoblast cells over the ICM which forms a multilayered structure extending anti-mesometrially as the extra-embryonic ectoderm and mesometrially as the ectoplacental cone. Thus morphogenesis could depend on comparatively simple processes like differential rates of cell division acting within mechanically constrained systems.

The analytical technique

The method of analysis used in this paper relies on reconstruction from serial histological sections and therefore has several limitations. Shrinkage or swelling due to the histological procedure could lead to artificial relocation of cells after fixation. However, a balanced fixative was chosen in order to minimize such effects (Baker, 1966) and it is considered unlikely that the method of counting nuclei would allow incorrect classification of cells. In most cases polar nuclei are markedly elongated tangentially, and polar cytoplasm shows a difference
in intensity of staining, so that clear distinction between ICM and polar trophodermal nuclei can be made. It did not prove feasible to distinguish between primitive endoderm and ectoderm in a quantitative manner and so these cell types, which share a common origin (Gardner & Papaioannou, 1975) were counted together as ICM.

The use of colcemid as a method of ‘collecting’ metaphases may be criticized since it is known that colchicine may lead to the death of rat foetuses after injection into pregnant females (Van Dyke & Ritchey, 1947). However, the short time of exposure of blastocysts to colcemid in the present experiments makes it unlikely that any deleterious effects would have appeared prior to fixation, especially since CT blastocysts contained the same number of dead cells as untreated controls. Moreover, when CT blastocysts aged 3 days 20 h.p.c. were transferred to the uteri of pseudopregnant recipients, live young were delivered at term and appeared completely normal (A.J.C. unpublished).

**Aspects of cell division in the blastocyst**

In the oldest blastocysts studied the rate of cell division in all regions is declining. This suggests a lengthening of cell cycle times towards implantation which is surprising since mitotic activity is more intense in embryos immediately after implantation (Snow, 1976). It therefore seems likely that the onset of implantation is associated with an increase in the rate of cell division which, if uniform throughout the polar and ICM regions, could lead to marked synchrony of cell division in the early egg cylinder. This point requires investigation.

The ratio of ICM cell number of BTCN falls throughout the blastocyst period. This has been interpreted as indicating that trophoderm cells are recruited from the ICM at least during early blastocyst development (Handy-side, 1978). However, data in Fig. 3B indicate that ICM and polar trophoderm do not differ greatly in their average rates of cell division, and Fig. 3C shows that there is considerable death in the ICM (see Appendix for further discussion). Consequently it is concluded that the fall in the ratio of ICM cell number to BTCN is consistent with the concept of separate ICM and trophodermal cell lineages from the time of blastocyst formation.

**Significance of cell death**

Although cell death is considerable in the ICM, and possibly in the trophoderm (see Appendix), of mouse blastocysts, dying cells appear to be isolated and necrotic zones are not found. In other developing systems localized regions of cell death have been implicated in morphogenesis (see Saunders, 1966) but the pattern of necrosis in the blastocyst makes it unlikely that cell death is serving a morphogenetic function. It is likely that processes of degeneration are initiated in cells some considerable time before such dying cells become morphologically distinguishable. So the peak of cell death in Fig. 3C may not reflect
an increase in the onset of cellular degeneration at this time, but at some earlier
time. On the basis of the present evidence therefore, it is difficult to speculate
about the role of cell death in mouse blastocyst development. Nevertheless it
must be borne in mind that during investigations of blastocyst protein synthesis
by two-dimensional gel electrophoresis (e.g. Johnson, Handyside & Braude,
1977) the pattern of spots seen on gels could include the protein products of
degenerating cells present during the labelling period.

APPENDIX

A semiquantitative analysis of the data was undertaken to ascertain whether
the observed rate of cell division in each region was compatible with the cell
number increase in the same region. Four blastocyst regions were considered:
mural, polar, combined mural plus polar, and ICM. Cell death was discounted
since the time taken for a cell to die is unknown (see Discussion). A comparison
of the rate of cell number increase with the rate of cell division was made for
each region by calculating ‘observed’ and ‘expected’ doubling times ($t_d$).
‘Observed’ $t_d$ was defined as the time taken for a particular blastocyst region
to double its cell number given the observed rate of cell number increase during
that time. ‘Expected’ $t_d$ was the time required for the same doubling of cell
number given the expected rate of cell number increase, which was calculated
from the mitotic index at that time. This method of analysis requires the data
to be plotted against age $p.c.$ rather than BTCN and so introduces an inaccuracy.
In order that the measured mitotic indices could be considered to represent the
whole of a given blastocyst region, it was necessary to assume that all blastocyst
cells divide, and that division is asynchronous. The latter assumption is not
strictly true (see Fig. 3B). The mitotic index measured for regions of blastocysts
 treated with colcemid for 2 h is an estimate of the number of cells in mitosis
at the time of colcemid injection plus the number of cells entering mitosis in
the ensuing 2 h. To find the rate of cells entering mitosis, the percentage of cells
expected to be dividing at any one time must be subtracted from the mitotic
index. Indices for NCT blastocysts give an estimate of the number of cells in
mitosis at any one time. However, NCT data were not available for all age
groups. So, average mitotic indices were calculated for all NCT blastocysts and
for CT blastocysts in the same age groups, and the ratio of the two average
mitotic indices was found separately for each region. This ratio was called the
‘correction fraction’. Each single mitotic index (for CT blastocysts) was then
corrected by subtracting the appropriate ‘correction fraction’, so giving cor-
crected mitotic indices ($M_{IC}$) which were estimates of the percentages of cells
entering mitosis in 2 h. Regional cell numbers and $M_{IC}$ values did not increase
logarithmically with age $p.c.$ Therefore it was necessary to compute best fit
lines for each set of data in order that rates of cell number increase could be
found for use in calculating doubling times. Lines of best fit for each of second,
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Fig. 4. Graphs to regional cell number against age post coitum (h) for (A) mural, (B) polar, (C) combined mural plus polar and (D) ICM regions. Circles indicate actual measurements. ▲—▲, the best-fit second order polynomial expression; ———, the best-fit eighth order polynomial expression for the mural data.

fourth, sixth and eighth order polynomial expressions were found by Chebyshev polynomial curve fitting using NAG library routines EO2ADA and EO2AEA run on an ICL 1906A computer. Higher order polynomial curves most closely fitted the fine detail of the data (e.g. Fig. 4A). However, it was felt that low order expressions more nearly resembled the biological situation because they smoothed out fluctuations in the data which may have been due to natural variation in small samples. Consequently second order expressions were used throughout (Figs. 4 and 5). ‘Observed’ doubling times were found at various ages between 82 and 109 h p.c. using gradients of the best-fit lines (Fig. 4 A–D) at each of these ages. ‘Expected’ doubling times were found by the formula:

\[ \text{‘Expected’ } t_d = \frac{100 \times 2}{\text{MI}_e} \text{ hours,} \]

using MI\(_e\) values obtained from the best-fit lines (Fig. 5A–D) at the same ages p.c. Table 2 shows the calculated ‘observed’ and ‘expected’ doubling times for the four blastocyst regions. These values were treated as matched pairs and subjected to Wilcoxon Matched Pairs tests. The application of this test is legitimate since the two sets of data are related, drawn from populations of similar characteristics, and it is both meaningful and desirable to rank the difference scores (Meddis, 1975). ‘Expected’ doubling times significantly
Fig. 5. Graphs of average mitotic index against age post coitum (h) for (A) mural, (B) polar, (C) combined mural plus polar and (D) ICM regions. Triangles indicate actual measurements. •—•, the best-fit second order polynomial expression.

Table 2. 'Observed' and 'expected' cell number doubling times (h) for the mural, polar, combined mural plus polar, and ICM regions at various times post coitum (see Appendix for explanation)

<table>
<thead>
<tr>
<th>Age post-coitum (h.)</th>
<th>Mural</th>
<th>Polar</th>
<th>Mural plus polar</th>
<th>ICM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$t_d$</td>
<td>$t_d$</td>
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<td>82</td>
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<td>83</td>
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Observed $t_d$ | Expected $t_d$ | Observed $t_d$ | Expected $t_d$ | Observed $t_d$ | Expected $t_d$ | Observed $t_d$ | Expected $t_d$ | Observed $t_d$ | Expected $t_d$ |
exceeded ‘observed’ doubling times in the mural region (T = 2, N = 17, P < 0.001) and in the trophectoderm as a whole (T = 0, N = 17, P < 0.001) and ‘observed’ doubling times exceeded ‘expected’ values in the polar region (T = 28, N = 17, P < 0.025). However, ‘expected’ and ‘observed’ doubling times did not differ significantly in the ICM (T = 53, N = 17, P > 0.05).

From these results it would appear that the polar region divides too fast and the mural region too slowly to be compatible with their own rates of cell number increase, while the rate of division of ICM cells approximately accounts for their rate of increase. The incompatibility of the rates of cell division and cell number increase in the combined mural and polar regions can be explained in two ways. The possibility that trophectoderm cells arise in the ICM has already been discussed and data presented above indicate that ICM cells are dividing no faster than can be accounted for by the rate of ICM cell number increase.

The alternative hypothesis is that some or all of the polar dead cells originated in the ICM, and were relocated prior to fixation of the blastocysts. Support for this idea comes from previous studies of dead cells in mouse blastocysts (Wilson & Smith, 1970; El Shershaby & Hinchliffe, 1974) which have failed to find trophectoderm cell death. This would lead to overestimation of trophectoderm cell number and ICM division rates and an underestimation of trophectoderm division rates and ICM cell numbers. In addition, from Table 2 it can be seen that the design of the significance test tends to obscure the fact that on average trophectodermal ‘expected’ doubling times exceed ‘observed’ doubling times by only a small amount. Consequently, a reclassification of polar dead cells as part of the ICM could lead to a non-significant difference between trophectodermal ‘expected’ and ‘observed’ doubling times, while the balance between ‘observed’ and ‘expected’ doubling times in the ICM would not be significantly altered.

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REFERENCES


Cellular proliferation in mouse blastocysts


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