

Cell redundancy in the zona-intact preimplantation mouse blastocyst: a light and electron microscope study of dead cells and their fate

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SUMMARY

In the zona pellucida-intact 95 h *post coitum* mouse blastocyst, electron-microscopic studies reveal the presence, as a part of normal development, of 1–2 dead cells lying free on the surface of the inner cell mass (ICM) or trophoblast cells, and of 4–5 dead cells phagocytosed by ICM cells. Such dead cells are electron-dense and show characteristic chromatopycnosis of the nucleus, and swelling of the endoplasmic reticulum. In addition, 1–2 digestive vacuoles with granular contents and myelin figures are found, principally in ICM cells, and corresponding with basophilic bodies observed with the light microscope. The results are interpreted as indicating that a relatively large number (i.e. a minimum of 6–8, or approximately 10%) of blastocyst cells die and are phagocytosed and digested, usually by the ICM cells, but probably also by trophoblast cells. This process does not, however, affect the future differentiation of the ingesting cell. Simultaneously a small number of epithelial cells adjacent to the blastocyst die, either singly or in small groups. These findings confirm the view that previous reports of penetration of the uterine epithelium by 'primary invasive cells' originating in the ICM were due to confusion between two separate groups of dead cells, namely the embryonic dead cells of the ICM and the single dead cells in the adjacent uterine epithelium, which appear to be phagocytosed by trophoblast cells following loss of the zona pellucida.

INTRODUCTION

A surprising feature of embryonic development is the regular appearance of cell death during specific phases of differentiation (reviewed by Glücksmann, 1951; Saunders, 1966; Saunders & Fallon, 1966). While much is known about the control and morphogenetic significance of cell death in the development of such organ systems as the chick limb (Hinchliffe & Ede, 1973; Hinchliffe & Thorogood, 1974) and tadpole tail (Weber, 1969), little is known about cell death in the early mouse embryo. Although the standard accounts of the histology and ultrastructure of implanting mouse and rat blastocysts (Snell & Stevens, 1966; Enders & Schlafke, 1967; Reinius, 1967; Mayer, Nilsson & Reinius, 1967) make no mention of the presence of dead cells, other workers

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have reported the presence in the implanting mouse egg (at about the time of loss of the zona pellucida) of basophilic bodies within inner cell mass (ICM) cells (Potts & Wilson, 1967; Potts, 1969). Recently Wilson & Smith (1970) have suggested these bodies might represent dead cells. At the same time, a small number of single dead cells appear in the uterine epithelium initially at 94–97 h *post coitum* (*p.c.*) (Finn & Lawn, 1968), well before the process of breakdown of the whole uterine epithelium around the implanting blastocyst at 120 h *p.c.* (Snell & Stevens, 1966; Potts, 1969). Considerable controversy surrounds the question of the basophilic bodies and dead cells in the preimplantation blastocyst. In his initial study in which the discovery of the basophilic bodies in the ICM was first reported, Wilson (1963) concluded that these represented specialized 'primary invasive cells' which appeared initially in the ICM and, following the shedding of the zona pellucida, migrated via the trophoblast cells into the uterine epithelium. This view was supported by Potts (1968, 1969), based on his interpretation of blastocyst ultrastructure; however, he expressed reservations about the cellular nature of the 'primary invasive cells'. Finn & Lawn (1968), in an electron-microscope study, suggested in contrast that the 'migration' was not from embryo to epithelium, but in the reverse direction, from epithelium to embryo, in fact consisting of trophoblast cells phagocytosing single dead epithelial cells which appeared at this time amongst their viable neighbours. Recently Wilson & Smith (1970) have accepted Finn & Lawn's interpretation. The question of dead cells in implantation is of considerable theoretical importance in view of the suggestion that primary invasive cells 'trigger the decidual reaction and epithelial degeneration' (Wilson, 1963; Potts, 1969) or that transfer of epithelial cell material may confer maternal immunological properties on the foetus (Wilson & Smith, 1970).

The present study was undertaken to resolve the problem of the formation and fate of the basophilic bodies in the zona pellucida-intact preimplantation blastocyst, which was examined at both light- and electron-microscope level principally for isolated dead cells and for stages of phagocytosis by both ICM and trophoblast cells. The uterine epithelium was also examined for cell death. A further report will analyse the autolysis of the uterine epithelium and related trophoblast phagocytic activity during implantation at 105–120 h *p.c.* (El-Shershaby & Hinchliffe, 1974).

MATERIAL AND METHODS

Mice were paired randomly overnight and the females examined at 10 a.m. on the following day for copulation plugs. Implantation sites were identified by injection of 0.25 ml of a 0.1% solution of pontamine sky blue 5BX in 0.9% saline into the tail vein of a mouse (Psychoyos, 1961) anaesthetized for 15 min by intra-peritoneal injection of nembutal at a concentration of 3 mg per 50 g body weight. A positive blue reaction was obtained first at 95 h *p.c.* Implan-

tion sites at 95 h and 105 h *p.c.* (assuming coitus took place at 1 a.m. on the night of mating) were fixed for electron microscopy. Eight implantation sites were examined at each of the two stages. The whole uterus was placed directly in cold 4% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4 for $\frac{1}{2}$ h, and the uterus then cut into pieces with a blue implantation site in the centre of each, and fixed for a further $2\frac{1}{2}$ h. (Attempts to open the uterine lumen to achieve more rapid fixation resulted in the dislodgement of the blastocyst, as reported by Potts (1969).) After washing in 0.1 M cacodylate buffer at pH 7.4 for 5 h, the pieces of uterus were postfixed in 1% osmium tetroxide in cacodylate buffer for 1 h, then washed in cacodylate buffer for 15 min, dehydrated and embedded in Araldite/epon mixture or in Araldite. Thin 1 μ m light-microscope sections stained in hot 0.2% toluidine blue, and ultrathin sections stained with 5% uranyl acetate and lead citrate, were prepared and examined.

RESULTS

At the time the pontamine sky blue reaction first appears, the blastocyst is located in the anti-mesometrial part of the uterine lumen. At 95 h *p.c.* about half of the blastocysts had an intact zona pellucida, but by 105 h all blastocysts were free from the zona pellucida. Only those 95 h *p.c.* blastocysts with the intact zona pellucida are described here, since its presence excludes the possibility that the dead cells or basophilic bodies are extra-embryonic in origin.

Isolated dead cells (one or two in each blastocyst) may be identified in the ICM or on the inner surface of the ICM or adjacent trophoblast cells (Figs. 1A, D; 2A). These dead cells are rounded, and at the ultrastructural level clearly show the process of chromatopycnosis (Glücksmann, 1951) involving concentration of the chromatic material of the nucleus and loss of the nuclear membrane (Fig. 2A). Cytoplasmic changes include increased electron-density (generally a sign of dehydration), gross swelling of the endoplasmic reticulum and ribosomal segregation. (McReynolds & Hadek (1972) refer to swollen endoplasmic reticulum as being a characteristic of ICM endoderm differentiation, but this takes the form of a wide-branching network, quite different from the grossly swollen form in dead cells (Fig. 2A).) Since these dead cells were formed in all blastocysts examined, in which mitotic figures could frequently be identified, it must be concluded that the death of ICM cells is a regular feature of normal development.

Within the same blastocyst, four or five basophilic bodies smaller in size than the isolated dead cells are often found in well-defined vacuoles in both ICM and trophoblast cells (Fig. 1B, D, E). Under the electron microscope, these bodies are clearly identified as dead cells with recognizable nucleus and cytoplasm which appear to have been phagocytosed by their viable neighbours. In Fig. 2B and C a dead cell (or possibly two) is found in large vacuoles and is shared between two embryonic cells. This cell has undergone further deterioration (as

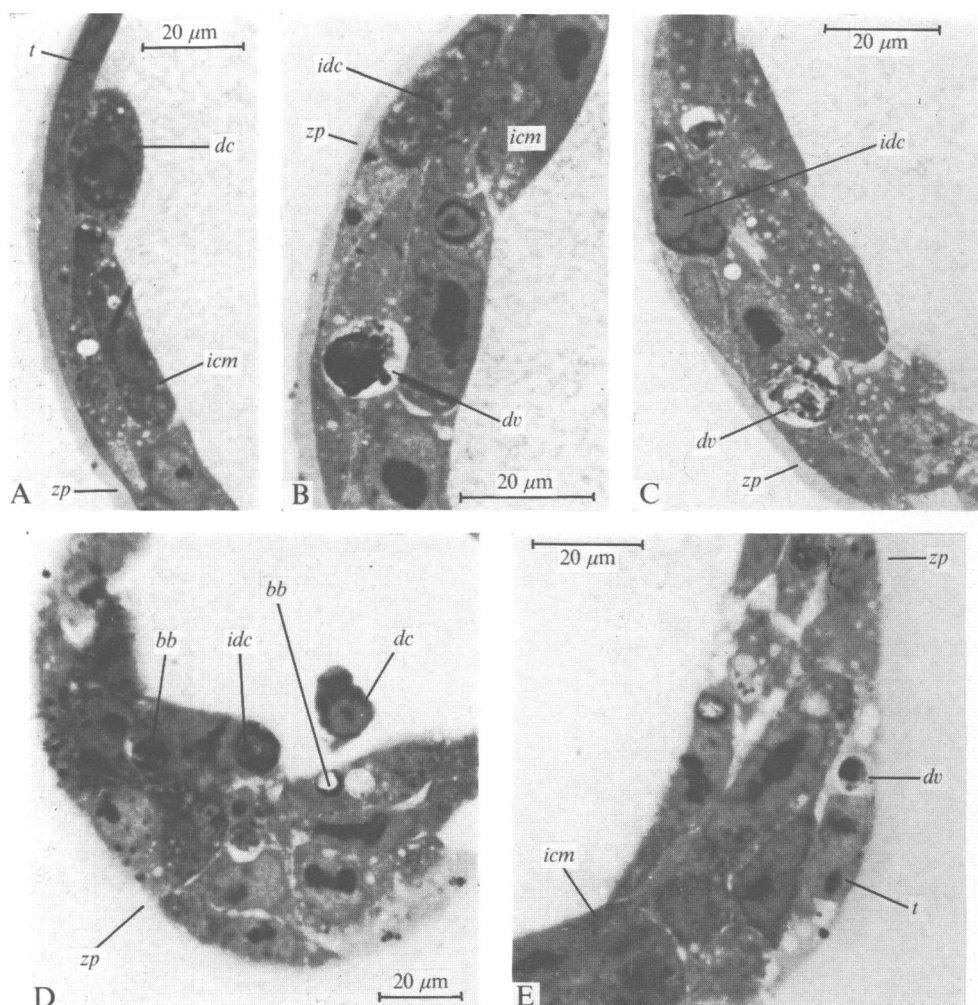


Fig. 1. Stages of cell death, phagocytosis and digestion in toluidine blue-stained $1 \mu\text{m}$ sections of 95 h *p.c.* zona pellucida-intact blastocyst. (A–C) represent different levels within the same blastocyst. (A) Isolated dead cell (*dc*). (B, C) Ingested dead cell (*idc*) and digestive vacuole (*dv*) in ICM cells. (D) Isolated dead cell (*dc*) on ICM inner surface, ingested dead cell (*idc*) and basophilic bodies (*bb*) in ICM cells. (E) Digestive vacuole (*dv*) in trophoblast cell. Inner cell mass (*icm*), trophoblast cells (*t*), zona pellucida (*zp*).

compared with Fig. 2A); the nucleus is fragmented and surrounded by a gap, and the cytoplasm shows absence of endoplasmic reticulum, and of ribosomes from certain regions, while in other areas they are segregated. Parallel lamellae, resembling those described by Enders (1971) in viable cells of the rat blastocyst, are also found in the cytoplasm (Fig. 2C).

ICM and trophoblast cells are also found with well-defined digestive vacuoles,

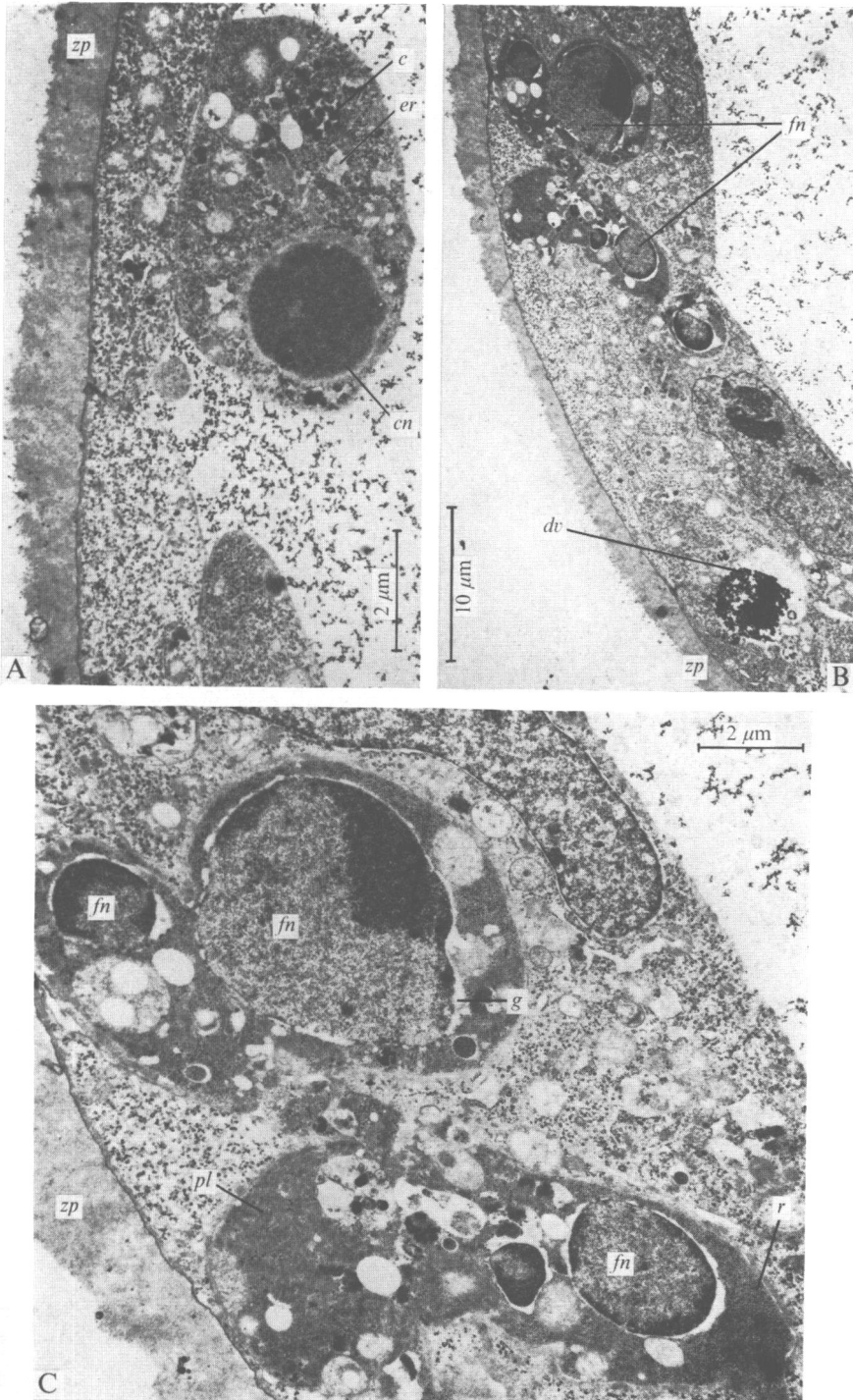


Fig. 2. Electron micrographs of stages of cell death, phagocytosis and digestion in 95 h *p.c.* zona pellucida-intact blastocyst. (A–C) are from the same blastocyst, which is illustrated at the light-microscope level in Fig. 1A–C. (A) Isolated dead cell; note chromatopycnotic nucleus (*cn*), swollen endoplasmic reticulum (*er*) and crystalloid material (*c*). (B, C) Phagocytosed dead cell (or cells); note fragmented chromatopycnotic nuclei (*fn*) separated from the cytoplasm by gaps (*g*), also parallel lamellae (*pl*) and aggregated ribosomes (*r*). Digestive vacuole with granular contents (*dv*), zona pellucida (*zp*).

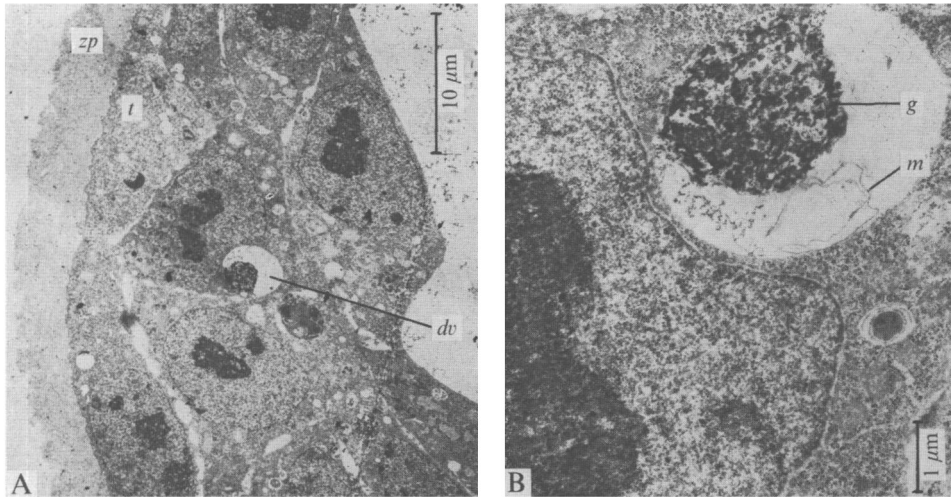


Fig. 3. Electron micrographs of digestive vacuole (*dv*) (a 'residual body') with granular contents (*g*) and myelin figures (*m*) in ICM cell. (B) detail of (A). Trophoblast cells (*t*), zona pellucida (*zp*).

with contents shown by the electron microscope to consist of granular material (without recognizable cell organelles) and myelin figures (Fig. 2 B; 3 A, B). These vacuoles are interpreted as 'residual bodies' (de Duve, 1963) with contents representing late stages in the digestion of phagocytosed dead cells.

While Wilson & Smith (1970) have previously interpreted basophilic inclusions in ICM cells as dead cells phagocytosed by neighbours, the present report is the first unequivocal evidence at the EM level of the presence of single isolated dead cells in the ICM and of the presence of dead cells complete with identifiable nucleus and cytoplasm within phagocytic vacuoles of ICM cells. Potts (1968, 1969) and Potts & Wilson (1970) have described ICM cells containing large electron-dense inclusions (with granular or lamellar contents) rather similar to the 'residual bodies' described above. The present authors believe that these inclusions in fact represent final-stage digestion of phagocytosed dead cells by viable ICM cells.

During the period when the zona pellucida is still present, in the majority (six out of eight) of implantation sites a few isolated dead or dying cells (1–4 dead cells) or small groups of dead cells staining intensely with toluidine blue are found in the surrounding uterine epithelium (Fig. 4 A–E). Such dead or moribund cells are found only in the uterine epithelium surrounding the blastocyst, and not in the epithelium a short distance away from the blastocyst. However, at 105 h *p.c.*, four of the eight sites examined showed between 2–5 isolated dead epithelial cells some distance away from, and mesometrial to, the implantation chamber. Epithelial dying cells are generally more electron-dense; there is condensation of the chromatin, particularly at the nuclear margin, the cytoplasm

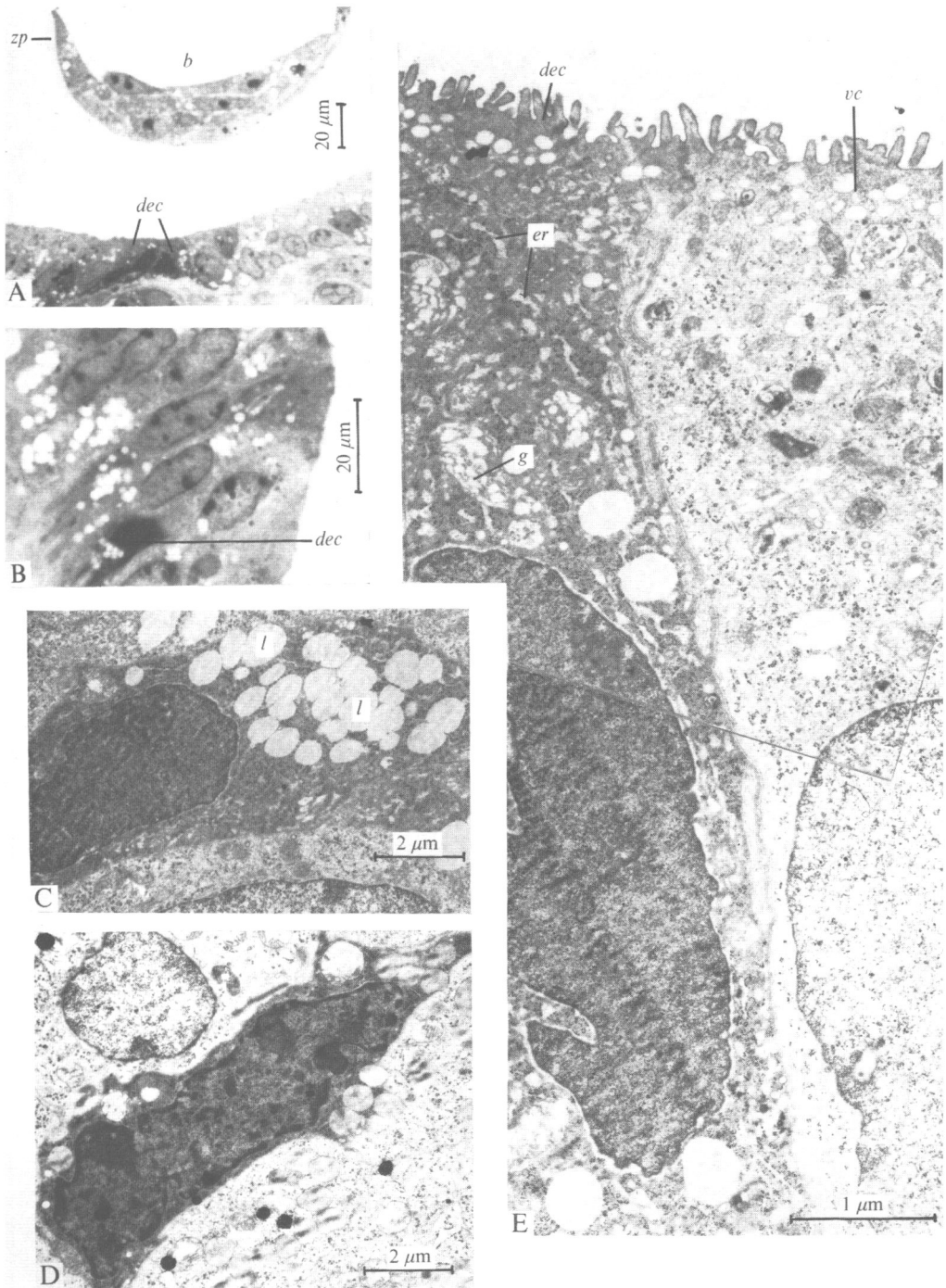


Fig. 4. Cell death in the uterine epithelium adjacent to 95 h *p.c.* zona pellucida-intact blastocysts. (A) Three adjacent dead epithelial cells (*dec*), blastocyst (*b*), zona pellucida (*zp*). (B) Single dead epithelial cell (*dec*). (C–E) Electron micrographs. (C) Dead cell illustrated in (B) showing increased electron density and lipid droplets (*l*). (D) Dead epithelial cell showing pronounced chromatopycnosis of the nucleus. (E) Dead epithelial cell (*dec*) and adjacent viable cell (*vc*), illustrated also in (A). The dead cell shows increased electron density, swollen endoplasmic reticulum (*er*) and Golgi body (*g*).

shows lipid accumulation, ribosome segregation, swelling of the endoplasmic reticulum and probably also of the Golgi body (Fig. 4C, E). At a later stage of deterioration, chromatin condensation is more advanced and the nucleus takes on an irregular form (Fig. 4D).

This appears to be the first report of degenerating epithelial cells around the zona pellucida-intact blastocyst. However, other studies have reported similar bodies in the epithelium, following loss of the zona pellucida, which appear to represent the same stages of early and advanced degeneration of single epithelial cells (Potts, 1968, fig. 17; Finn & Lawn, 1968, figs. 1-3; Wilson & Smith, 1970, fig. 6).

In the 105 h *p.c.* blastocysts, no isolated dead cells were found in the ICM region, but digestive vacuoles with basophilic contents were found in both ICM and trophoblast cells.

DISCUSSION

This paper reports evidence of cell redundancy and death in the ICM before zona pellucida loss. The source of these cells, whether from ICM or trophoblast, or from both, is not clear. By adding the number of recognizable isolated and phagocytosed dead cells to the number of large digestive vacuoles thought to represent late phases in digestion of dead cells, we arrive at a total of six or eight dead cells. It is possible that this number may represent a peak of cell death in the blastocyst, occurring at about 95 h *p.c.*, since (i) Wilson & Smith (1970) and Potts & Wilson (1967) report only one basophilic body to a section at 86 h and 90 h *p.c.* respectively, and since (ii) in the later 105 h *p.c.* blastocyst only late digestion phases rather than isolated dead cells are found. It should be noted that the estimated number of six or eight dead cells represents a *minimum*, since all dead cells are not necessarily present at the same time, depending on the speed of their digestion and the length of the period of time over which they are produced.

At this time the blastocyst consists of about 64 cells, of which only approximately 13 make up the ICM (Graham, 1971). Although the regulative capacity of the blastocyst at this stage is well known (Gardner, 1972) it is surprising that the blastocyst should lose as many as six or eight cells at this stage, and it is therefore important to consider the reasons for such relatively massive cell redundancy. ICM cell death in the blastocyst coincides with the time at which ribosomal RNA synthesis increases sharply and the embryonic genome first becomes active (McLaren, 1972), and at which differentiation has reached the stage when separated trophoblast and ICM cells first show behavioural differences (Gardner, 1972). Cells failing to differentiate normally may thus be the source of the dead cells. Linked with this is the possibility that such dead cells may represent an accumulation of cells having relatively minor chromosomal abnormalities, insufficient to interfere with their division, but sufficient to prevent them responding to a subsequent differentiation stimulus (Glücksman,

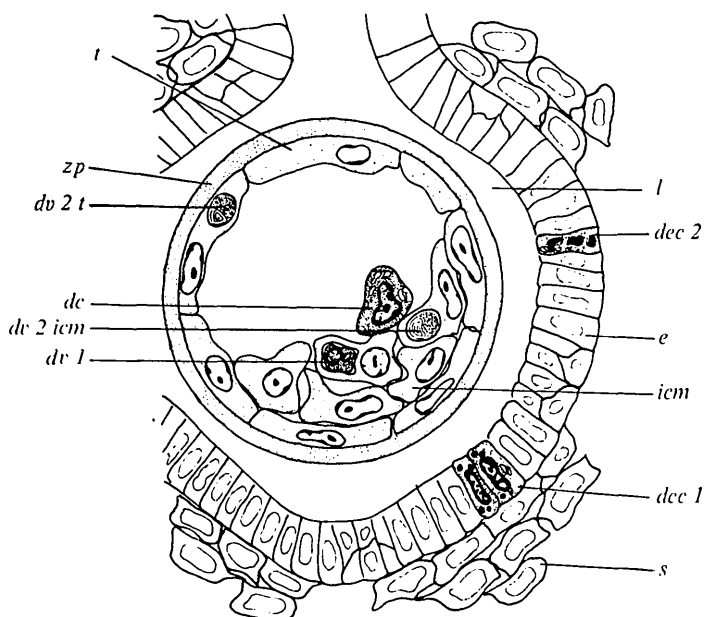


Fig. 5. Diagrammatic illustration of cell death processes in the 95 h *p.c.* mouse blastocyst prior to zona pellucida loss.

dc, Dead cell in ICM *dec*, dead epithelial cell, early phase (1), or late phase (2); *dv 1*, digestive vacuole, early phase; *dv 2*, digestive vacuole, late phase, in ICM cell (*icm*) or in a trophoblast cell (*t*); *e*, epithelial cells of uterus; *icm*, inner cell mass; *l*, lumen of uterus; *s*, stroma; *t*, trophoblast; *zp*, zona pellucida.

1965; Forsberg & Källén, 1968). Such damaged cells may fail to respond to the developmental mechanisms governing differentiation of ICM and trophoblast cells, or to the inductive mechanism recently analysed by Gardner (1972) in which the ICM governs trophoblast differentiation.

Whatever may be the reason for the appearance of isolated dead cells in the ICM, the subsequent stages are clear: the dead cells are phagocytosed, primarily by neighbouring ICM and probably also by trophoblast cells, as summarized in Fig. 5. The light-microscope observations of basophilic bodies in trophoblast cells are in conflict with Wilson & Smith's statement (1970) that at the time inclusions are found in ICM cells there are no such inclusions in trophoblast cells. Following phagocytosis, the dead cells appear to be digested within the phagosomes, whose contents range from dead cells complete with cytoplasm and nucleus through partially digested but recognizable cell organelles, to myelin figures and granular remnants. The interpretation of these results as indicating a process of ingestion and digestion of dead cells by their neighbours is identical with that put forward in relation to cell death in the mesenchyme of the developing rat (Ballard & Holt, 1968) and chick limb (Dawd & Hinchliffe, 1971). However, there is no indication that phagocytosis affects the future

pathway of differentiation of the ingesting cell, which does not appear to differentiate into a macrophage (the normal fate of ingesting cells in chick and rat embryo limbs). It is predictable that the process of digestion of the dead cells involves the participation of lysosomal enzymes (de Duve, 1963, 1964) and a study of the lysosomal enzyme acid phosphatase showed the presence of enzyme-rich vesicles in the ICM before the zona pellucida had been lost (Smith & Wilson, 1971). Smith & Wilson interpret these enzyme-rich vesicles as spontaneously degenerating embryonic cells, but it is more probable that they represent intense lysosomal enzyme activity within the phagocytic vacuoles of viable neighbouring cells during digestion of ingested dead cells, rather than activity within the isolated dead cell itself (Ballard & Holt, 1968; Weber, 1969; Dawd & Hinchliffe, 1971).

The question of the origin of basophilic bodies in the trophoblast cells is important in relation to the 'primary invasive cell' question. Wilson & Smith (1970) carried out an autoradiographic study in which labelled blastocysts were cultured *in vitro* or were transferred to pseudopregnant host uteri, but they concluded that their results did not enable them to establish clearly the maternal or embryonic origin of these trophoblast inclusions. The presence of the zona pellucida in the blastocysts described in this paper firmly establishes the embryonic origin of these bodies in the trophoblast cells.

The presence of the intact zona pellucida is important in relation to a second question. A small number of individual epithelial cells around the blastocyst die at about 93–100 h *p.c.* and it is claimed that these are phagocytosed by trophoblast cells (Finn & Lawn, 1968; Wilson & Smith, 1970). Do the trophoblast cells actually kill the epithelial cells, or do they merely phagocytose them after death? Wilson & Smith (1970) claim it is the specific activity of the trophoblast cells which kills the individual uterine epithelial cells. However, the results reported here in which individual dead cells appear in the uterine epithelium before dissolution of the zona pellucida barrier make it clear that epithelial cell death cannot be due to any direct contact with trophoblast cells, which may however transmit some factor toxic to epithelial cells through the intact zona pellucida. But such a factor would be expected to affect all adjacent epithelial cells and it is difficult to account for the appearance of individual dead cells in the epithelium at this time. Since such dead cells are only found in the epithelium near the site of implantation, they cannot be attributed to a general process of epithelial cell turn-over and replacement, and it may be that these cells represent a specific response (of unknown significance) to the presence of the implanting blastocyst. This view is supported by McLaren's evidence (1968) that such degenerating epithelial cells (or 'W-bodies') characterize post-lactational or oestrogen-induced implantation, but are not found in the uterine epithelium surrounding a delayed blastocyst.

However they may be formed, such cells are clearly candidates for phagocytosis and digestion by the trophoblast cells, after zona pellucida dissolution.

Electron micrographs interpreted as representing ingestion of isolated dead epithelial cells by trophoblast cells at 105 h *p.c.*, following zona pellucida loss, will be reported in a second study (El Shershaby & Hinchliffe, 1974) of autolytic degeneration of the uterine epithelium in implantation. The authors follow Finn & Lawn (1968) in their interpretation, now also accepted by Wilson & Smith (1970), that it is this process of trophoblast ingestion of dead individual epithelial cells which has given rise to the previous reports of 'W-bodies' (Finn & McLaren, 1967) or of 'primary invasive cells' in transit in the epithelium (Wilson, 1963; Potts & Wilson, 1967). It should be stressed that this process takes place before the general breakdown around the implantation site of the *whole* uterine epithelium at 120 h *p.c.*, a process which has been shown to be an inherent property of the uterus during implantation chamber formation and the decidual cell reaction, and not due to trophoblast attack (Finn & Hinchliffe, 1965).

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