Ultrastructural analysis of preimplantation lethal yellow \((A^y/A^y)\) mouse embryos

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SUMMARY

Embryos recovered at 62 and 80 h post coitum from reproductive tracts of yellow \((A^y/a)\) and black \((a/a)\) female mice mated to \(A^y/a\) males were examined ultrastructurally to define the developmental defect of lethal \(A^y\) homozygotes.

No abnormalities were observed in five embryos from control matings \((\% a/a \times \% A^y/a)\). Of 24 morulae and blastocysts from \(A^y/a \times A^y/a\) matings, six were observed to possess some morphological aberration. Two of the six abnormal embryos were morulae and contained isolated blastomeres which had developmental features typical of younger embryos; remaining cells of these embryos were normal. The third, an early blastocyst, contained a degenerating trophoblast cell; other cells of this embryo were also abnormal but not in an advanced stage of degeneration. The fourth abnormal embryo (late cleavage stage) was in an advanced stage of degeneration affecting all blastomeres. Finally, the remaining two abnormal morulae had a unique nucleolar morphology and an unusual abundance of intra-cisternal A particles.

Presumably, one or more of the six abnormal embryos from \(A^y/a \times A^y/a\) matings were \(A^y\) homozygotes. However, no single ultrastructural alteration characteristic of \(A^y/A^y\) embryos was found.

INTRODUCTION

Many descriptive studies have been conducted to investigate the cause of death of \(A^y\) homozygotes (Ibsen & Steigleder, 1917; Kirkham, 1919; Robertson, 1942; Eaton & Green, 1962, 1963; Cizadlo, 1976); however, the primary \(A^y\) defect remains obscure. Pedersen (1974) reported that homozygous \(A^y\) embryos developed abnormally \textit{in vitro} during the preimplantation period. Subsequent studies have confirmed that a population of experimental embryos (presumably \(A^y\) homozygotes) are developmentally retarded at 2- and 4-cell stages (Pedersen & Spindle, 1976; Johnson, 1977). Calarco & Pedersen (1976) examined the ultrastructure of preimplantation embryos from \(A^y/a \times A^y/a\) matings; following their recovery from uterine tracts, cleavage-stage embryos, morulae, and blastocysts were cultured until identified as \(A^y/A^y\) mutants by the criteria of Pedersen (1974) and then examined.

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The present study was undertaken to discover morphological manifestations of lethal $A^v$ expression. Since the onset, duration, and variability of lethal $A^v$ expression in $A^v/A^v$ embryos are as yet undetermined, we examined pre-implantation embryos from cleavage to definitive blastocyst stages, without prior culture.

**MATERIALS AND METHODS**

Mice derived from C57BL/6J-$A^v/a$ or -$a/a$ stock obtained from The Jackson Laboratory were used in this study. Virgin yellow ($A^v/a$) or black ($a/a$) females (70–130 days of age) were placed with $A^v/a$ males and inspected daily for vaginal plugs. Copulation was assumed to have occurred at 02.00 and hours post coitum (h.p.c.) were calculated from that time. Embryos were recovered at 62 and 80 h.p.c. and immediately prepared for electron microscopy (Enders, 1971).

A total of 70 embryos were prepared from four experimental matings ($A^v/a \times A^v/a$, 31 embryos, 7-8 ± 0-9 embryos per female) and five control crosses ($a/a \times A^v/a$, 39 embryos, 7-8 ± 0-9 embryos per female). Twenty-four of 31 experimental and 5 of 39 control embryos were randomly chosen for ultrastructural examination from the embedded material. Twenty of the 24 experimental embryos examined were derived from the 62 h.p.c. recovery group, while the remainder were obtained at 80 h.p.c. Of control embryos examined, three were fixed at 62 h.p.c. and two at 80 h.p.c. About 93 % of the embryos recovered at 62 h.p.c. were either 4-cell (27 %) or 8-cell (66 %) stages; at 80 h.p.c. 35 % were morulae and 65 % blastocysts.

In contrast to the study of Calarco & Pedersen (1976), embryos in this investigation were not cultured but were prepared immediately upon recovery for electron microscopy. Also, in our study no attempt was made to select $A^v/A^v$ mutants for observation; instead, embryos were randomly chosen according to the following procedure. Plastic blocks were first observed to see if an embryo could be found anywhere in the block. If an embryo was found, that block was included in a pool. From these, blocks were simply drawn like drawing numbers from a hat. Once chosen for electron microscopy embryos were not staged
Ultrastructural analysis of A²/A² mouse embryos
within blocks prior to ultramicrotomy. Following electron microscopy ultrastructural characteristics described by Enders & Schlafke (1965), Szolozi (1971), and others were used to classify embryos at various stages.

RESULTS

Observations on embryos derived from $A^v/a \times A^v/a$ matings revealed two classes of embryos. Members of the first class appeared to be ultrastructurally normal and were presumed to have either $A^v/a$ or $a/a$ genotypes. Of 24 experimental embryos, 18 fell into this category, as also did all 5 control embryos. Features of these presumed $A^v/a$ and $a/a$ embryos are described below.

Nuclei of normal morulae generally had one large, rounded, dense primary nucleolus (Fig. 1a), while late morula and blastocyst nuclei (Fig. 1c) contained small, dense areas (pars amorphae) surrounded by a diffuse network (reticulation or nucleonema). Several experimental embryos had one or two large, dense nucleoli surrounded by a compact, rather than a network-type of granular zone (Fig. 1b). Further, these nuclei had diffuse, scattered dense granular areas throughout the nucleoplasm (arrows, Fig. 1b), similar to fibrillo-granular bodies described by Hillman & Hillman (1975). Mitochondria in earlier stages appeared more spherical (Fig. 2a), while those of older embryos were more elongate (Fig. 2c); mitochondria of morulae often possessed inner membranes which were reflected inward at several points to form large vacuoles (Fig. 3c). Membrane-limited dilated profiles were observed which were frequently associated with mitochondria (er, Fig. 2a). In early blastocysts the enclosed volume within such endoplasmic reticular cisternae decreased markedly (Fig. 3d). Every embryo examined contained crystalline aggregates associated with endoplasmic reticulum. As described by Enders & Schlafke (1965), crystals were frequently attached to endoplasmic reticulum forming right angles between the membrane and the crystalline axis (Fig. 3d).

A doughnut-like structure described by Enders & Schlafke (1965) in the guinea-pig blastocyst and by Calarco & Brown (1969) and Calarco (1975) in

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Fig. 2. Electron micrographs of ultrastructurally normal embryos from $A^v/a \times A^v/a$ matings.

(a) Crystals, endoplasmic reticulum with few attached ribosomes (er), and spherical mitochondria (m) within a morula. Arrow indicates crystal which appears cross-hatched.

(b) Outer membrane of morula blastomere. Cross-sections of microvilli (arrowhead) have an inner clear zone surrounded by a ring of spaced granules, which in turn is enclosed by a slightly more electron-dense zone. Cortical differences in electron density are also apparent in longitudinal sections.

(c) Portions of three cells bordering on the blastocoele within an early blastocyst. Zone of clear cytoplasm encircles the large lipid droplet (L). Mitochondria (m) are less spherical than at earlier developmental stages, but are vacuolated. Specialized areas of contact are present along cell interfaces.
Ultrastructural analysis of $A^y/A^y$ mouse embryos
the mouse was seen (Fig. 3a, b). This structure, which has been described as virus-like and termed an intracisternal A particle (IAP), was seen more frequently in younger embryos but was also found in those with a well-developed blastocoel. Lipid accumulations, a common feature of all embryos, were found most frequently in association with mitochondria, jigsaw bodies, and multivesicular aggregates. Jigsaw or J-bodies originally identified by Calarco & Brown (1969) were seen as a universal cellular constituent. Structures previously described as degradation and myelin bodies were seen in all embryos. Cytoplasmic vesicles were sometimes grouped into what Calarco & Brown (1969) termed multivesicular aggregates. These aggregations consisted of larger central vesicles each surrounded by a single layer of many smaller vesicles (MVA, Fig. 3a).

Free cellular surfaces generally possessed small microvilli. An arrangement of fine filaments could be seen in some sections (Fig. 2b); certain cross-section profiles of microvilli displayed a clear central zone surrounding by a homogeneous ring. Invaginations of the cell surface were frequently observed on the free surface and between cells. Some sections showed an electron-dense area (coated pit) at the concave surface of the invagination; occasionally, fibrous strands were seen projecting into the cytoplasm from this surface as described by Enders & Schlafke (1965; fig. 14F).

**Presumed mutants** (Ay/Ay) from Ay/a × Ay/a matings

Unusual structures or structural arrangements were observed in six embryos from experimental (Ay/a × Ay/a) matings. One very degenerate cleavage-stage embryo had large intercellular spaces, very irregular cell membranes, and an exceedingly crowded cytoplasm. The cytoplasm of this embryo appeared very granular and contained large degradation bodies. J-bodies were abnormally clear. Nucleolar reticulations were present but appeared fragmented. A second embryo (blastocyst) possessed one vacuolated trophoblast cell which was

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*Fig. 3. Electron micrographs of ultrastructurally normal embryos from Ay/a × Ay/a matings.*

(a) Organelles of blastomere of morula. Mitochondria are spherical; arrow indicates mitochondrion in which cristae lie parallel to the outer membrane. Asterisks indicate endoplasmic reticulum containing many intracisternal A particles. Aggregation of lipid droplets (L) is typical; cytoplasm in the area of droplets is less dense than elsewhere in the cell. Fibrous strands are scattered throughout the cytoplasm. Area within rectangle is enlarged in (b).

(b) Enlargement of (a) showing granular endoplasmic reticulum containing doughnut-shaped intracisternal A particles. Vacuolated mitochondria are also seen.

(c) Portions of mitochondrion (m) and concentric membranes of myelin body (mb) within a cell of a morula. Asterisk indicates vacuole within mitochondrion whose contents resembles background cytoplasm.

(d) Flattened endoplasmic reticulum with many attached ribosomes (arrow) and crystals (c) within an early blastocyst. No intracisternal A particles were present. A portion of a multivesicular aggregate is seen in the lower right of the figure.
Ultrastructural analysis of A\textsuperscript{v}/A\textsuperscript{v} mouse embryos
degenerating (Fig. 4a). Also within this specimen was a blastomere which contained a large, empty vacuole (Fig. 4b). Another cell showed a large, organelle-free cytoplasmic projection (Fig. 5a). Two morulae were examined each of which contained an isolated blastomere; however, the remainder of the cells appeared normal. The isolated blastomeres contained very condensed cytoplasmic material. Two morulae had a strikingly increased number of IAP within the endoplasmic reticulum. They also had large nucleoli with far less than usual reticulation (Fig. 1b), and scattered dense areas as seen in some of the presumed normal embryos. Further, one of these embryos was clearly binucleate and had an isolated blastomere (Fig. 5b). In all other respects, these embryos appeared normal.

**DISCUSSION**

Observations on normal nucleolar morphogenesis in presumed A\textsuperscript{v}/a and a/a morulae agree well with previous studies (Calarco & Brown, 1969; Hillman & Tasca, 1969). Nucleoli in two experimental embryos classified as abnormal appeared retarded for their chronological age. These embryos also contained large numbers of IAP which should occur only in younger embryos (Calarco & Szollosi, 1973). Apparently, these embryos were developmentally retarded, but positive identification as lethal homozygotes (A\textsuperscript{v}/A\textsuperscript{v}) was not possible. However, since one of these embryos also had binucleate cells, the possibility that these were normal is lessened.

Blastocysts appeared to contain more crystalline material than morulae. Embryos containing flattened endoplasmic reticulum with large numbers of attached ribosomes had the most crystals, while embryos with dilated endoplasmic reticulum with few attached ribosomes and more numerous IAPs contained the least. Interestingly, crystals were found in all six experimental embryos classified as abnormal and in isolated blastomeres which were in an advanced stage of degradation. Also, presence of numerous crystals in degenerating isolated blastomeres indicates that these cells had developed normally, at least in those biochemical pathways which support crystal synthesis, to an advanced preimplantation stage.

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Fig. 4. Electron micrographs of an ultrastructurally abnormal (presumed A\textsuperscript{v}/A\textsuperscript{v}) embryo from A\textsuperscript{v}/a \times A\textsuperscript{v}/a matings.

(a) Portions of three cells of an abnormal early blastocyst. A degenerating blastomere is present between the zona pellucida at the top of the figure and two apparently normal cells at the bottom. At this magnification intracisternal A, particles (IAP) appear crowded and dense. Membrane of degenerating cell is irregular. Crystalline arrays (c), lipids and multivesicular aggregates within the degenerating cell are normal in appearance. Organelles of other cells observed from this embryo appeared normal.

(b) Abnormal blastomere with large intracellular vacuole within the same early blastocyst as (a). Organelles of this cell are ultrastructurally normal. However, cytoplasm of other blastomeres within this embryo were less dense than their neighbors and had abnormally large, organelle-free areas.
Ultrastructural analysis of $\alpha^2/\alpha^+$ mouse embryos
Fig. 5. Electron micrographs of ultrastructurally abnormal (presumed A/V/AV) embryos from A/V/a x A/V/a matings.

(a) Abnormal organelle-free cytoplasmic projection within the same early blastocyst as Fig. 4. Note the difference in density between the projection and the portions of the two cells seen at the right. Cell outlines were irregular in this embryo.

(b) Binucleate cell of abnormal morula. Nuclear morphology is similar to the nucleus seen in Fig. 1b. Arrow indicates area rich in intracisternal A particles. Flattened, electron-dense membranes are present near lipid droplets.
Ultrastructural analysis of $A^v/A^v$ mouse embryos

The probability of our experimental sample containing all $A^v/a$ or $a/a$ embryos for this investigation is 0.001 ($3/4$ to the 24th power). Therefore, it is almost certain that at least one of the observed embryos was of the $A^v/A^v$ genotype. With the expected segregation, the sample examined would, on the average, contain six lethal homozygous embryos. Six embryos were found with abnormal characteristics, but due to the diversity of observed abnormalities, it seems doubtful that these all represent $A^v/A^v$ embryos. Since previous investigators (Kirkham, 1919; Robertson, 1942; Eaton & Green, 1962, 1963) found the expected ratio at or shortly after implantation, abnormalities at the morula-to-early blastocyst stage would probably be ultrastructural rather than gross. Of the abnormal embryos examined, those which are most likely to be the lethal homozygotes are either those with isolated degenerating cells or those which are lagging in development. These findings agree with the identification by Pedersen (1974) of developmentally arrested embryos or those with some degenerating, excluded cells as the $A^v/A^v$ embryos in culture. Also, data that $A^v$ homozygotes lag behind litter-mates as early as 2- and 4-cell stages have been reported by Pedersen & Spindle (1976) and confirmed by Johnson (1977).

The description of the presumptive $A^v/A^v$ embryos by Calcarco & Pedersen (1976) agrees with ultrastructural observations reported in the present study. In both reports, isolated blastomeres had ultrastructural features characteristic of embryos at an earlier developmental stage, i.e. round vacuolated mitochondria, IAPs, rounded nucleoli, few endoplasmic reticular cisternae, and few polysomes.

Whatever the biochemical alterations caused by $A^v$, they do not appear to manifest themselves in any obvious morphological way. Although excluded blastomeres containing immature organelles may be characteristic of $A^v$ homozygotes, Johnson (1977) has also observed a 13.8% (12/87) incidence of excluded blastomeres in preimplantation embryos of reciprocal yellow by black ($A^v/a \times a/a$) control matings. Once excluded and therefore removed from inductive influences of the intact embryo, such blastomeres could fall out of developmental synchrony and differentiate at a slower rate than non-excluded blastomeres.

In summary, although 25% (6/24) of the experimental embryos examined possessed one or more abnormalities, no single ultrastructural aberration unique to $A^v$ homogygotes was discovered. Neither the present study nor the ultrastructural examination of Calcarco & Pederson (1976) provide data on precise morphological events caused by primary gene expression in the lethal yellow ($A^v/A^v$) mouse.

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