Ultrastructural observations of lethal yellow (Ay/Ay) mouse embryos

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SUMMARY

Ay/Ay embryos were identified by the presence of large excluded blastomeres (Pedersen, 1974) and examined cytologically and ultrastructurally. Cell organelles, inclusions and junctions in the excluded blastomeres were compared with those of non-excluded cells of Ay/Av embryos and control embryos. Excluded blastomeres always had the fine structural characteristics of earlier developmental stages and may have arrested at the 4- to 8-cell stage or slightly later. Interior cells (inner cell mass) were observed in all mutant blastocysts. Non-excluded cells of Ay/Av embryos were normal until degenerative changes appear in the late blastocyst stage. The mode of action of the +Ay gene was not determined, but evidence from this study and others indicates that the effects of +Ay gene action occur over a wide range of time in early cleavage and implantation.

INTRODUCTION

Embryos homozygous for the yellow allele (Ay) of the agouti locus show characteristic abnormalities at the morula stage during growth in vitro (Pedersen, 1974) and die in vivo during implantation (Kirkham, 1919; Robertson, 1942; Eaton & Green, 1962). Observation of living Ay/Ay embryos at the morula and blastocyst stages suggests that they are capable of the initial differentiation of inner cell mass and trophoblast, in spite of the reduced embryonic mass that results from the earlier arrest and exclusion of some blastomeres (Pedersen, 1974). This paper presents ultrastructural and light microscope observations that were made to determine whether presumed Ay/Ay blastocysts actually possess an inner cell mass, and to examine the characteristics of the arrested and remaining blastomeres of the abnormal embryos.

MATERIALS AND METHODS

Source of mice. Male and female C57 BL/6J-Ay/a mice were obtained from the Jackson Laboratories, Bar Harbor, Maine.

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Source of embryos. Male \( A^v/a \) mice were caged with groups of five spontaneously ovulating \( A^v/a \) females, which were checked for copulatory plugs the following morning (day 1 of pregnancy). Embryos were flushed from the oviducts or uteri with modified Hanks' balanced salt solution (Goldstein, Spindle & Pedersen, 1975) on day 3 at the 4- to 8-cell stage or the next day as morulae and blastocysts. The proportion of abnormal embryos (presumed \( A^v/A^v \)) from this cross was approximately 22% and did not differ significantly from the published value of 23.9% (Pedersen, 1974).

Embryo culture. Embryos were cultured in the standard egg culture medium of Biggers, Whitten & Whittingham (1971) modified to contain 102 mM NaCl and 3 mg/ml bovine serum albumin (Pentex). Identification of abnormal, presumably \( A^v/A^v \) embryos, was based on the criteria and characteristics previously described (Pedersen, 1974).

Ultrastructure and cytology. Embryos were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer and postfixed in 2% osmium tetroxide in 0.1 M phosphate buffer. Embryos were rapidly dehydrated through increasing concentrations of ethanol and embedded in Epon 812 (Luft, 1961). Serial thick and thin sections were cut through presumptive \( A^v/A^v \) embryos and control embryos. The thick sections were stained with 1% toluidine blue in order to locate abnormal regions of the embryos for fine structural study. Contrast of the thin sections was enhanced by treating the sections with saturated uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963). Sections were examined in a Hitachi HS-8 electron microscope.

Observations

A total of 17 abnormal \( (A^v/A^v) \) embryos, including 8-cell, morulae and blastocysts, were examined by light microscopy and electron microscopy. \( A^v/A^v \) embryos can be easily recognized at the morula or blastocyst stage with the light microscope and usually exhibit one or more large arrested blastomeres (Pedersen, 1974). This process of blastomere exclusion occurs \textit{in vivo} and \textit{in vitro}. In control matings, abnormal embryos occur at a frequency of 8.8% (Pedersen, 1974), but do not exhibit arrested, excluded blastomeres. In the present study we examine morulae and blastocysts which developed from the 4- to 8-cell stage \textit{in vitro} and some \( A^v/A^v \) blastocysts which developed \textit{in vivo}. There are no obvious fine structural differences between \( A^v/A^v \) embryos developing \textit{in vivo} or \textit{in vitro}, although they both differ from normal embryos in the same litters and from normal embryos of random-bred mice.

The most noticeable difference between \( A^v/A^v \) embryos and normal ones is the presence of one or more large blastomeres which are excluded from the embryo proper (Figs. 1, 2). These large blastomeres have no particular 'mutant' characteristics \textit{per se}, but always have the fine structural characteristics of earlier developmental stages, and usually are similar to 8-cell blastomeres.
Non-excluded blastomeres appear normal ultrastructurally until degenerative changes appear in the late blastocyst stage. The relative stage of development of blastomeres in this study is based on the organelle changes which normally occur during early cleavage (Enders & Schlafke, 1965; Calarco & Brown, 1969; Hillman & Tasca, 1969) and are detailed below.

The excluded cells in the $A^v/A^v$ blastocysts are on the periphery of the embryo, i.e. external to the trophoblast cells, although in one case an arrested blastomere was in the blastocoel (Fig. 2). The peripheral excluded cells are occasionally joined to one trophoblast cell by a junctional complex (Figs. 3, 4) but are not joined to other excluded cells by such junctions. Junctional complexes normally form between exterior cells at the morula stage (approximately 16 cells) (Calarco & Brown, 1969; Enders, 1971).

In the $A^v/A^v$ blastocysts, intracisternal A particles (IAP) are seen only in excluded cells, not in normal blastocyst cells. Crystalloid inclusions are present in excluded and normal blastomeres but seem more numerous in the former (Fig. 5). Both IAP and crystalloids are normally present in large numbers at the 4- to 8-cell stage, but IAP are rarely present by the blastocyst stage (Calarco & Brown, 1969). C particles are not present in the $A^v/A^v$ blastocysts or normal blastocysts in this study. Older, expanded blastocysts were not examined. Fibrous inclusions are also quite common in excluded cells (Fig. 3) but are rarely present in the embryo proper after the blastocyst stage is reached. It has been reported that these fibrous arrays, which are common to all young cleavage stages in the mouse, are reduced or disappear by the blastocyst stage (Burkholder, Comings & Okada, 1971; Enders, 1971). The mitochondria of excluded blastomeres of $A^v/A^v$ embryos are smaller, more spherical and exhibit larger intracistral vacuoles than do mitochondria of the non-excluded cells (Figs. 3, 5). An increase in mitochondrial size and reduction of intracristal vacuoles occurs during cleavage of normal mouse embryos (Enders & Schlafke, 1965; Calarco & Brown, 1969; Hillman & Tasca, 1969; Stern, Biggers & Anderson, 1971). In addition, the peripheral filamentous layer seen beneath the plasma membrane in control and non-excluded blastocyst cells is either absent (Fig. 3) or diminished (Fig. 4) in excluded blastomeres. Nucleoli of excluded cells (Fig. 6) exhibit granules but in general are round and have large dense fibrillar cores characteristic of younger cleavage stages (Calarco & Brown, 1969; Hillman & Tasca, 1969; Szollosi, 1971). Furthermore, they do not achieve the elongate, irregular appearance seen in normal blastocyst nucleoli. In addition, excluded cells contain many fewer polysomes and less rough endoplasmic reticulum (Figs. 3, 5) than do normal morula or blastocyst cells.

There is some variation in the relative stage of development of the excluded blastomeres, however. The excluded cells in two of the $A^v/A^v$ embryos examined have more polysomes, fewer fibrous arrays and fewer mitochondrial intracristal vacuoles (Fig. 4) than the excluded cells described above and may be develop-
Fig. 1. Photomicrograph of a 1 μm section through an $A^s/A^v$ morula embedded in Epon and stained with toluidine blue. Arrow indicates excluded blastomere. × 640.

Fig. 2. Photomicrograph of a 1 μm section through an $A^v/A^v$ blastocyst. There are three excluded cells visible including one within the blastocoele. $I$, inner cell mass. Asterisk indicates area illustrated in Fig. 3. × 640.

Fig. 3. Electron micrograph of section adjacent to Figure 2. Note the lack of polysomes and rough endoplasmic reticulum in the excluded cells ($EX$). $J$, junction between excluded cell and blastocyst; $F$, fibrous inclusions; $B$, blastocoele; $M$, peripheral microfilaments. × 18920.
mentally advanced beyond the 8-cell stage. One embryo, identified by light microscopy as abnormal at the 8-cell stage, revealed by electron microscopy one blastomere, which was larger than the others with the characteristics of a 4-cell blastomere, most notably a large spherical, agranular nucleolus with a very small nucleolonema.

Interior cells were identified in all mutant blastocysts (Fig. 2). In order to determine whether these cells were from the trophoblast or inner cell mass, they were examined ultrastructurally for the presence of junctions typical of trophoblast cells (Calarco & Brown, 1969; Enders, 1971). Since they did not form junctional complexes with each other, or with exterior cells they were presumed to be inner cell mass cells. Light and electron microscopic observations suggest, however, that the number of interior cells in $A^u/A^u$ embryos was lower than in control embryos.

Some cytoplasmic degradation is observed in normal blastomeres and intact excluded blastomeres, as well as in control embryos. In some cases the excluded blastomeres have disintegrated by the blastocyst stage. When $A^u/A^u$ blastocysts are cultured overnight, they collapse and extensive cytoplasmic degeneration is observed with the electron microscope.

**DISCUSSION**

Several investigators have described the changes which subcellular organelles undergo during early mouse development (Enders & Schlafke, 1965; Calarco & Brown, 1969; Hillman & Tasca, 1969; Enders, 1971; Stern et al. 1971; Szollosi, 1971). These changes have been used to characterize the developmental stage of abnormal blastomeres of $A^u/A^u$ embryos. The retention of IAP, fibrous inclusions, and small vacuolated mitochondria, and the depression of the ribosomal system (rounded nucleoli, few polysomes or rough endoplasmic reticulum), confirms that one or more blastomeres usually arrest at the 8-cell stage or later, but occasionally may arrest earlier. The remaining blastomeres of the $A^u/A^u$ embryo form a small blastocyst and appear to develop normally until degeneration at the late blastocyst stage begins. In fact, $A^u/A^u$ embryos collapse and die without hatching from the zona pellucida although limited trophoblast outgrowth occurs if the zona is removed experimentally (Pedersen, 1974). Some of the arrested cells in $A^u/A^u$ embryos form tight junctions with the non-arrested cells, a trait of morula cells (Calarco & Brown, 1969; Enders, 1971) which suggests that some cell functions may continue in the arrested blastomeres. What determines the early sensitivity of several, but not all of the blastomeres to $+A^u$ gene action during cleavage, however, remains an intriguing question.

Since interior, non-trophoblast, cells are present in $A^u/A^u$ blastocysts, we conclude that these embryos are capable of inner cell mass and trophoblast differentiation in spite of their earlier defects. No obvious deterioration of inner
Figures 4-6
Observations of lethal yellow mouse embryos

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cell mass cells was observed at the early blastocyst stage, and the reduced number of inner cells, compared with normal embryos, is probably a result of the small size of $A^y/A^y$ embryos, rather than preferential early death of inner cells. However, the inner cell mass of $A^y/A^y$ embryos does not survive during blastocyst culture after experimental removal of the zona pellucida, although at least some trophoblast cells survive and grow out (Pedersen, 1974). This in vitro effect could be due to the initially smaller number of inner cells in $A^y/A^y$ blastocysts. However, reduced embryonic mass is not sufficient to account for death of presumed $A^y/A^y$ embryos in vivo because normal fetuses can be obtained when one blastomere of a mouse embryo is destroyed at the 2-cell stage (Tarkowski, 1959). Alternatively, the sensitivity of inner cell mass cells at the post-blastocyst stage to effects of $+A^y$ gene action may be due to their normally rapid proliferation compared with trophoblast cells (Barlow, Owen & Graham, 1972). A similar interpretation has been suggested for the differential sensitivity of inner cell mass and trophoblast development in vitro after acute X-irradiation at the morula and blastocyst stages (Goldstein et al. 1975).

The $A^y/A^y$ embryos' early defects described here and their growth potential during post-blastocyst culture (Pedersen, 1974) indicate that the effects of the $+A^y$ allele occur over a wide range of time between early cleavage and implantation. However, neither the precise time of action of the $+A^y$ gene nor its primary effect on the embryo can be determined from the present study.

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Fig. 4. Portion of an $A^y/A^y$ blastocyst. The appearance of the excluded blastomere (EX) suggests arrest later than the 8-cell stage. In fact it is similar ultrastructurally to the adjacent blastocyst cell, both showing few polysomes. B, blastocoele, J, apical junctional complexes. × 5940.

Fig. 5. Portion of an excluded, arrested blastomere from an $A^y/A^y$ blastocyst illustrating the mitochondria, crystalloids (C) and paucity of polysomes. × 20110.

Fig. 6. Nucleolus of cell excluded in the blastocoele of Fig. 2. Note the rounded shape, the condensed nucleolonema and the fibrous regions (F). × 9860.


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