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EFFECTS OF LETHAL FACTORS ON THE EARLY DEVELOPMENT OF MOUSE EMBRYOS

By

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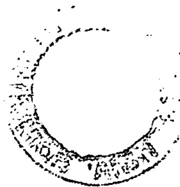

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Summary

Investigations have been performed into the mode of action of three recessive lethal factors in the mouse, which cause the death of homozygotes during early embryonic development.

Embryos homozygous for yellow (A^y), which die at the time of implantation, are found to have become typically abnormal and necrotic after a short period of delayed implantation, indicating that inability to implant in the uterus is not the primary cause of death. In culture, A^y/A^y embryos fail to outgrow normally in standard or modified media. The relationship observed between abnormal morphology prior to culture and subsequent failure to outgrow, confirms other reports that the homozygote embryos may be affected significantly earlier than the effective lethal phase. In vitro aggregation experiments show no evidence of "rescue" with respect to outgrowth capability, and it is concluded that lethality, of the trophoblast at least, is cell-autonomous.

Development of embryos homozygous for oligosyndactylism (Os) is arrested during primary implantation. Air-dried preparations indicate that many cells become blocked in metaphase. Histological investigations reveal that trophoblastic giant cells are relatively unaffected by the action of the mutant gene and this is confirmed during culture over the lethal phase; outgrowing trophoblastic giant cells seem morphologically normal although reduced in number, while the inner cell mass becomes totally degenerate. It is proposed that the form of endoreduplication undergone by giant cells renders them refractory to the metaphase block.

An histological study shows that embryos homozygous for tail-

Summary (contd)

short (Ts) become abnormal as morulae, although in some instances a small amount of development, including cavitation, may continue to take place during the following day. Similar behaviour is exhibited in culture. Autoradiographic investigations of Ts/Ts morulae imply that some RNA synthesis persists even after they have become phenotypical identifiable.

DECLARATION

I declare that this thesis describes the results of original work performed by me, and was written entirely by me.

SECTION 1

GENERAL INTRODUCTION

<u>The Effects of Lethal Factors on Development</u>

The term "lethal factor" is applied in practice to any genetic modification which results in the death of the organism concerned prior to the reproductive stage. Dominant lethals are by their very nature eliminated immediately from a population and are thus not only of little eugenic significance, but are also unsuitable for systematic developmental analysis. Recessive lethals, however, may be easily maintained and are therefore of great value as tools for investigating the physiology of development. Such recessive lethal or semi-lethal factors, the majority of which cause abnormalities of morphology or pigmentation in the heterozygous condition, have been reported to occur at over fifty autosomal loci in the mouse genome. At several of these loci, including <u>albino</u> (c), <u>short ear</u> (se), and most notably <u>brachyury</u> (T), multiple lethal alleles or pseudo-alleles exist which cause death throughout a spectrum of developmental stages and show complex complementation patterns indicating that these regions encompass more than one functional unit (Gluecksohn-Waelsch et al.1974; Russell 1971; Bennett et al.1975).

The majority of recessive lethals cause death at the perinatal stage. However this does not accurately reflect the underlying distribution of morphogenetic disturbances which are the true causes of death; rather the demands of an independent existence constitute a superficial threshold beyond which severe abnormality is no longer compatible with life. Consequently the respective phenocritical phases of recessive lethal factors are to be found distributed more or less continuously throughout development, although displaying a bias toward

the later stages of embryogenesis, which presumably reflects an increase in the number of genes deployed as the structure and functions of the embryo become more complex.

The recessive lethality of genetic factors whose effective lethal phase is significantly earlier than parturition is commonly first detected from the analysis of breeding data by the absence of the homozygote class, and it is of significance that virtually all mutations known to affect the developmental pattern in the mouse during the first week of gestation are indeed lethal. It may be concluded from this fact that any disruption of the early processes of embryogenesis is likely to be so devastating in consequence that development is rapidly brought to a halt. Since the events of the first few days of development in the mouse are concerned with securing a suitable environment in which cells can subsequently proliferate to form the definitive embryo, any factor which interferes with the mechanisms of either implantation or primary differentiation clearly must be extremely deleterious. In short, disturbances of early embryogenesis appear always to be lethal.

This is demonstrated elegantly by the actions of the much celebrated recessive t-alleles, which fall into discrete complementation groups, each connected with defects in differentiation most noticeably of the ectoderm or ectodermal derivatives at specific stages spanning early development (see Bennett 1975 for review). These, together with a number of other lethal factors, reveal just how critical the early stages of differentiation are to the subsequent viability of the embryo. t^{12}/t^{12} embryos arrest as morulae (Smith 1956), and although a degree of cavitation has been reported to occur in some cases (Hillman et al.

1970), it seems that the differentiation of blastomeres into clearly delineated inner cell mass and trophectoderm does not occur; as shown in Section 4, embryos homozygous for tail-short (Ts) are afflicted at a somewhat similar stage. Failure to consolidate connections with the uterus during implantation also inescapably halts development as seen with t^{w73}/t^{w73} blastocysts in which the trophoblast and ecto-placental cone are deficient in the invasive properties required for successful implantation (Spiegelman et al.1976). Similarly, yellow (A^y) reduces the number of primary invasive cells in the trophoblast (Eaton and Green 1963), although there is now considerable doubt as to whether this is a cause or an effect of developmental arrest (Pederson 1974; see also Section 2). Progressing a stage further, one of the lethal "alleles" at the albino locus c^{6H} (Gluecksohn-Waelsch et al.1974) has been shown, amongst other effects, to greatly impede proliferation of the ectoplacental cone (Lewis et al.1976), presumably as a result of interfering with the interactions between trophoblast and inner cell mass which seem to regulate the differentiation of this tissue (Gardner and Johnson 1972). Embryos homozygous for t^0 group alleles are capable of implanting normally, but appear unable to undergo the differentiation required for the production of separate embryonic and extraembryonic ectodermal components in the egg cylinder (Gluecksohn-Schoenheimer 1940); an informal report suggests also that trophoblastic giant cells may become abnormal (Lewis 1973). t^{w5}/t^{w5} embryos are able to implant and develop an initially normal egg cylinder, but the embryonic portion rapidly degenerates (Bennett & Dunn 1958). Continuing yet one more step in embryogenesis, three mutant factors - t^9,

blind (Bld) and one of the short-ear deficiencies (se^1) are all known to disrupt the differentiation of mesoderm via the primitive streak. In t^9 homozygotes, the small numbers of cytologically abnormal cells emerging from the primitive streak are incapable of establishing a functional mesodermal layer (Bennett and Dunn 1960; Spiegelman and Bennett 1974), while in Bld/Bld (Vankin 1956) and se^1/se^1 (Dunn 1972) egg cylinders, failure of mesoderm development is accompanied by specific abnormalities in the behaviour of endodermal and ectodermal cells respectively. Mutant alleles, whether recessive or dominant, acting on embryos beyond this stage are not always lethal, as exemplified by heterozygotes for tail-short (Ts) in which abnormalities of the blood-islands within the yolk-sac are clearly visible as early as 8 days post coitum, but which are usually capable of surviving into adulthood (Deol 1961).

It is thus evident that disturbances occurring in any of the developmental steps taken during early differentiation are likely to lead to the death of the embryo. Beyond this period various aspects of organogenesis are able to occur in relative isolation and consequently may have less immediately drastic consequences.

It is worth bearing in mind that the word "lethal" in the context discussed is nevertheless a relative term, the use of which should be confined to a specific set of genetic and environmental conditions. Gluecksohn-Waelsch (1963) points out that there can be no sharp distinction drawn between lethal factors and those compatible with life, since the borderline between vitality and mortality is extremely fine such that the effect of genes on development may be very significantly altered

according to the conditions obtaining. The semi-lethal t-alleles, for example, are highly variable in expressivity with respect to not only the absolute viability of the individual but also the time at which inviable embryos are arrested, presumably reflecting minute environmental differences. Similarly tail-short, in the heterozygous state, becomes a semi-lethal or even a complete lethal when introduced into certain mouse strains (Morgan 1950), thus demonstrating the influence of genetic background on gene action. Ultimately, since selection acts on phenotype rather than genotype, lethality must be regarded as arising from the joint interaction of a particular genetic factor and its specific environment.

The Action of Lethal Factors at the Cellular Level

Three basic ways may be considered by which death at the cellular level can be brought about directly by gene malfunction, either at structural or regulatory sites within a given locus:

(a) Inability to replace or supplement exhausted or insufficient stocks of a vital compound.

(b) Inability to manufacture normally a new enzyme or structural component required for a specific function of differentiation or development.

(c) Accumulation to toxic levels within the cytoplasm of compounds which act as antimetabolites or otherwise interfere with development.

Type "a" is most easily envisaged as operating in the early stages of development when the embryonic genome is required to assume control of metabolic processes. The exact time at which this occurs is

undetermined and may be a gradual procedure, although good evidence that the mouse genome is active to some extent during early cleavage comes from the demonstration of paternally derived gene products. Brinster (1973) has detected paternal-type GPI at the 8-cell stage, and the results obtained by Chapman et al.(1976) indicate that the gene for beta-glucuronidase may be transcribed even earlier. Paternal-type cell surface antigens have also been shown to be present at this time (Muggleton-Harris and Johnson 1976) and high molecular weight RNA synthesis is clearly apparent from the 2-cell stage (Woodland and Graham 1969; Knowland and Graham 1972). Nevertheless, such evidence only indicates that some embryonic gene expression can occur during early cleavage, and the debate over the existence of stored maternal messengers in mammalian embryos is yet unresolved, since their presence has proved difficult to demonstrate unequivocally (for reviews see Wolf and Engel 1972; Epstein 1975). Inhibitors of RNA synthesis such as actinomycin D and alpha-amanatin both severely interfere with early development (Mintz 1964a; Skalko and Morse 1969; Golbus et al.1973; Warner and Versteegh 1974), but it must be conceded that this is hardly surprising in the light of findings which indicate that the mouse embryonic genome is at least partially functional at this time. It may, therefore, be possible for genetically inviable embryos, even with the most basic latent metabolic inadequacies, to develop normally with the aid of maternally synthesised RNA until such time as embryonic gene function becomes a necessity.

A type "b" situation has been suggested as the cause of lethality in embryos homozygous for the recessive t-alleles (see Bennett 1975).

Specific antigens (F9), demonstrated immunologically to be present on cells of cleaving embryos, have been found to be altered in certain t mutants (Kemler et al.1976), although not in a sequential fashion corresponding to their respective phenocritical phases. It is thus proposed that the T-complex is responsible for the production of a series of cell surface antigens which govern cell to cell recognition systems during early development. According to this hypothesis, the alterations which occur in these surface components in homozygous t embryos seriously affect the interactions between neighbouring cells required for normal differentiation. Mutations of this type, whether disrupting structural or enzymatic properties, generally may be expected to be highly stage-specific since their effects will be directly related to particular phases of embryogenesis.

Type "c" events may be regarded as a form of self-poisoning by the embryo. This may be brought about by a subtle change in the structure of a metabolically active gene product, such as the substitution of one amino acid for another within an enzyme close to the active site, as a result of a mutation at the structural locus. Under certain conditions this could then possess antimetabolic properties, for example by retaining substrate specificity but with altered activity, thus seriously disturbing biochemical equilibria within the cell. Alternatively, imbalances in the intra-cellular concentration of a given compound, possibly arising from mutation of a regulatory site in the genetic material, could be equally deleterious. Such a system has been tentatively proposed as operating in the case of yellow (A^y) (Wolff 1971). Auto-toxicity of this sort might be expected to be less acutely stage-specific than the other

types of lethality discussed, since accumulation of deleterious compounds could be a gradual process subject to modification by the background genetic and environmental milieux.

Lethal factors may be further categorised into those which directly affect all cells within the organism - i.e. non-specific, and those which cause death as a result of their action on a particular group of cells - i.e. cell or tissue specific. Especially with early acting lethals it is often difficult to determine whether the failure of a given group of cells to differentiate in the normal pattern is the root cause of death, or whether the onset of degeneration throughout the entire cell population of the embryo is merely coincident with the stage at which this differentiation would normally take place. This is exemplified by the two conflicting theories concerning the mode of action of the t-alleles. As mentioned above, these lethals have been interpreted as primarily affecting ectodermal derivatives (Bennett 1975), for it is commonly the differentiation of this tissue type which first appears abnormal in histological studies of the homozygotes in utero. More recently, however, electron microscopy (Hillman et al.1970; Hillman and Hillman 1975; Nadijcka and Hillman 1975) together with investigations in vitro (Wudl et al.1977) have cast doubt upon this hypothesis, at least with respect to certain of these alleles, since the morphology and behaviour of other types of cells suggest that the action of this group of mutations may be considerably less specific than was previously thought. Similarly with yellow (A^y), the trophoblast specificity which has been attributed to the lethal action of the allele by Eaton and Green (1962; 1963), now seems in question (Pederson 1974; see also Section 2). Mutations which affect

equally all the cells of the embryo are likely to be those involving basic metabolic defects and therefore to be very early acting. Consequently, it is not surprising that the majority of recessive lethals studied in the mouse have been identified as cell or tissue specific, such as splotch (Sp) which affects developing neural tissue (Auerbach 1954) and dominant spotting (W) which acts via the haemopoetic system to cause death from macrocytic anaemia (Mintz and Russell 1957; Russell et al.1953).

Lastly, lethal factors may be characterised according to whether or not their effects may be influenced by the constitution of their immediate environment in chimaeras, mosaics, or explantation experiments. Absence of "rescue" of mutant cells by a normal environment in these situations indicates that the effect of the factor involved is cell-autonomous. In cell specific lethals, where death in many parts of the embryo is allophenic (i.e. lethality arises as a secondary consequence of the developmental inadequacies of other cells), some successful chimaerism or explantation will normally be possible. W/W ⟷ +/+ chimaeras can thus grow into perfectly viable adults, and indeed W/W cells may predominate in number over wild-type, with the exception of the blood where cells are found to be exclusively of wild-type origin (Mintz et al.1971). Some cells hemizygous for the sex-linked lethal jimpy (jp) have also been shown to be viable in chimaeras with wild-type (Eicher and Hoppe 1973). Using cultured explants, it has been demonstrated that various tissue types of embryos homozygous for brachyury (T) can survive and proliferate long after death would normally have occurred in the intact embryo (Ephrussi 1935); similarly, cells from t^{w18}/t^{w18} embryos may survive considerably beyond the normal

time of lethality in ectopic implants (Artzt and Bennett 1972) or in culture (Wudl et al.1977). Some degree of "rescue" may even be possible for autophenic characters (i.e. developmental inadequacies which are a direct consequence of the cell's own genic expression); explanted kidney rudiments from embryos homozygous for Danforth's short tail (Sd) show considerable differentiation when isolated in culture, although development is almost entirely suppressed in vivo, possibly as a result of asynchronous growth (Gluecksohn - Waelsch and Rota 1963; Gluecksohn - Waelsch 1963).

Non-specific lethals, on the other hand, are more likely to be entirely cell-autonomous since, by definition, all cells of the embryo are directly affected. It is nevertheless possible that, under certain circumstances, a basic biochemical deficiency in a potentially inviable cell might be remedied by the passage of the requisite metabolite from neighbouring metabolically competent cells. Mintz (1964 c) has been unable, however, to demonstrate any sign of "rescue" in cultured $t^{12}/t^{12} \longleftrightarrow +/+$ chimaeric embryos, a fact which has been taken in support of the argument that this early acting mutation is a non-specific lethal. The techniques of culture and chimaera production in mammals are able greatly to facilitate the investigation of the way in which lethal factors operate at the cellular level, in a field which was previously restricted, in the main, to lower orders of animals.

Without a full understanding of the exact nature and chromosomal basis of a recessive lethal factor, it is not easy to determine the precise biochemical origin of the lethality. Why, for example, should a particular mutation be lethal at an early stage of development in homozygotes, while producing only minor and seemingly unrelated effects at an

entirely different stage in heterozygotes? Kacser (1976) has concluded that recessivity of genetically determined enzyme deficiencies is a necessary consequence of enzyme systems in general. This being so, it seems likely that recessive lethality, at least in those cases where enzyme deficiencies are concerned, is often a direct result of the primary metabolic lesion. Since the viability of heterozygotes is often entirely unaffected during the lethal phase of the homozygotes (see Luning 1975), the dominant characteristics which commonly accompany recessive lethal factors (semidominants) may therefore occur as a consequence of secondary effects on the biochemistry of the cell, such as alterations in pool sizes of compounds involved in different metabolic pathways. Such secondary effects are much more likely to be susceptible to the influence of the general genetic or environmental background than is the complete deficiency of a vital enzyme; this is compatible with the marked variation in penetrance and expressivity often observed in the characteristics of the heterozygotes. In this type of system, genetic modifiers of expressivity with respect to the dominant effects are unlikely to be capable of increasing the viability of homozygotes, since they exert their influence on events which are remote from the primary metabolic disturbance.

According to the concept of "unity of gene action", pleiotropic effects of lethal factors may nevertheless be able to act as useful pointers to the underlying biochemical defect. For example, investigations into apparently unconnected multiple enzyme deficiencies caused by lethal alleles at the albino locus (Gluecksohn-Waelsch et al.1971; Erickson et al.1968; Thorndike et al.1972) led to the discovery of ultrastructural

abnormalities in the intracellular membranes which are thought to be the common localised sites of these enzymes (Trigg and Gluecksohn-Waelsch 1973). Oligosyndactylism (Os) , which causes the lethality of homozygotes during early development by blocking cell division (Van Valen 1966; see also Section 3), also results in digital fusions of the heterozygotes. A tantalising link between these diverse effects exists, therefore, in reports of syndactylism being induced in the developing limbs of the toad (Tschumi 1954) and the chick (Kieny 1975) by mitotic poisons. Yellow (A^y), in addition to its recessive lethality, produces a vast array of pleiotropic effects in the heterozygotes. Quite apart from its action on coat colouration, this allele influences such disparate characteristics as tumour growth (Heston and Deringer 1947; Heston and Vlahakis 1961; Vlahakis and Heston 1963; Deringer 1970), obesity (Fenton and Chase 1951; Carpenter and Mayer 1958), serum insulin levels (Wolff and Reichard 1970) and dental morphology (Leamy et al.1971). Wolff (1971), who has extensively studied the action of yellow together with its allelic counterpart viable yellow (A^{vy}) , has attempted to explain all the effects of this factor in terms of an imbalance of sulphydryl groups (SH) . Although his hypothesis has never been substantiated, it provides an excellent example of the method by which pleiotropism may be used to gain an insight into aberrant metabolic processes. Clearly, however, pleiotropic effects will only be of value as diagnostic aids to the investigation of lethal factors, if the genetic lesion involved is restricted to a single functional unit. Caution must consequently be exercised in interpreting this type of data, especially where extensive deletions are suspected.

The Genetic Basis of Lethality

Ultimately, the effect of lethal factors must be traced back to the level of the biochemistry surrounding the genetic material itself. Several loci in the mouse display series of "alleles" which produce a variety of developmental effects, not all of which are necessarily lethal. The complexity of genetic complementation between these "alleles" has led to the conclusion that several discrete functional units of the chromosome are involved. Complementation patterns at the T-locus enable the lethal t-alleles alone to be divided into 6 groups (Bennett 1975), and Russell (1971) has worked out a linear series of 8 or 9 chromosomal units within the dilute/short-ear region.

Similarly, recessive lethals at the albino locus (c) are found to fall into four complementation groups (Gluecksohn-Waelsch et al.1971). At the agouti locus too, Russell (cited by Wallace 1965) has reported 18 independent spontaneous and induced mutations, of which 3 were recessive lethals. Alleles at this locus show exceedingly intricate dominance relationships with respect to coat colour, which has led Wallace (1965), in support of an earlier suggestion of Pincus (1929), to conclude that several closely linked loci must be involved. This concept is supported by instances of crossing-over apparently taking place between these "alleles", including a reported recombination rate as high as 0.5% between the two lethal factors A^y and a^x (Russell et al.1963; see also Wallace 1954). The alternative explanation that these comparatively rare events are the result of spontaneous mutation cannot, however, be completely ruled out, and this interpretation has been given by Dickie (cited by Wallace 1965) to explain the appearance of

agouti (A) in non-agouti (a) stocks.

The chromosomal basis of lethal factors at all these loci remains somewhat obscure, particularly since methods such as complementation mapping can be misleading with respect to the physical layout of mutational sites within the gene. The complementation behaviour of the numerous short-ear and albino lethals, however, strongly suggests that at least some of these are small deficiencies. The suppression of crossing over along a significant region of the chromosome, which accompanies most of the lethal t-alleles (Lyon and Phillips 1959; Dunn et al.1962), led to speculation that chromosomal rearrangements are involved, and indeed Geyer-Duszynska (1964) claims to have observed regions of mispairing in pachytene chromosomes of mice heterozygous for certain t-alleles. It must be added, nonetheless, that subsequent investigators have been unable to support this finding (Bennett 1964; Munigle, cited by Bennett 1975; Dev et al.1971).

When suitable techniques have become available for examining selected regions of the mammalian genome at the molecular level, it is certain that such complex loci will play an important part in investigating the pattern of gene regulation during development.

Early lethals have, for many years now, demonstrated the involvement of the embryonic genome in early mammalian development - an occurrence which has only recently been detectable by alternative, sophisticated techniques. In particular they are able to shed light on the occult spheres of cellular interactions and inductive influences, which have tended to fall evasively between the reductionist analyses of the

molecular biologist on the one hand, and the morphological observations of the histologist on the other. Unique opportunities are presented for relating changes at the gross level of embryogenesis and histogenesis with specific events at cellular and molecular levels.

It is with these aims in mind that the following investigations have been undertaken into three early acting recessive lethal factors in the mouse - *yellow* (A^y) , *oligosyndactylism* (Os) and *tail-short* (Ts).

SECTION 2

A STUDY OF EMBRYOS HOMOZYGOUS FOR YELLOW (A^y)

INTRODUCTION

Numerous alleles affecting coat colour have arisen at the agouti locus, either spontaneously or radiation-induced, which by altering the relative proportions of black (eumelanin) and yellow (phaeomelanin) pigment in the hair shaft produce colours ranging from pure yellow (A^y), through agouti (A or +), to pure black (a^e extreme non agouti) (See Wolfe and Coleman for review). Several of these alleles have been briefly reported to be lethal when homozygous including lethal non-agouti (a^x) (Russell et al.1963) and recently non-agouti lethal (a^l) (Phillips 1976), but in most cases little is known of their mode of action in this respect. The exception, however, is yellow (A^y), the most dominant member of the series with regard to coat colour, which has been the subject of extensive investigation.

In 1905 Cuenot recorded that he had been unable to obtain any yellow mice which would breed true, and observing that litters from yellow x yellow crosses tended to be rather small, subsequently suggested that "yellow eggs" fail to be fertilized by "yellow sperm" (Cuenot 1908). A more detailed breeding experiment by Castle and Little (1910) revealed that approximately two-thirds of mice produced from yellow matings were themselves yellow, and confirmed Cuenot's suspicion that litters were smaller. Since the average size of such litters was not quite as low as the 75% which is statistically expected from the absence of the entire homozygote class, it was suggested that this could be accounted for by an increase in the general viability of the remaining embryos due to the less crowded conditions *in utero*. Kirkham (1917), investigating the time of death of the mutant homozygotes, discovered one or more

degenerating embryos amongst the morulae and blastocysts of all uteri from *yellow* females pregnant by *yellow* males. In a subsequent report (Kirkham 1919) he observed that these abnormal embryos, distinguishable by their shrunken appearance, crowded cells and small blastodermic vesicle, survived sufficiently long to induce a decidual swelling before being rapidly destroyed by phagocytes. It was also noted that the numbers of such embryos present was in reasonable agreement with the Mendelian expectation, for *yellow* homozygotes, of 25%. Ibsen and Steigleder (1917), examining uteri at 13-19 days *post coitum*, claimed to have found two types of abnormal embryos in females from *yellow* x *yellow* matings - a large group which failed to progress beyond the implantation stage (consistent with Kirkham's findings) and also a smaller group which became arrested at the thirteenth day of gestation. A curious feature of this report is the apparent state of preservation of the former group of embryos, from which it was concluded that the *yellow* homozygote does not perish "but merely ceases to develop after a certain stage has been reached and then remains more or less stationary till parturition. While this paper is frequently cited as being confirmatory to Kirkham's investigation, it seems more likely that the similarity of the author's respective interpretations is merely coincidental and that Ibsen and Steigleder were in fact observing some unrelated phenomenon.

A major study by Robertson (1942) confirmed beyond doubt that the death of *yellow* homozygotes, as small vacuolated balls of cells, occurs at the time of implantation, although he observed a less vigorous local uterine response, with the epithelium remaining intact. In contrast to Kirkham, however, he was unable to detect any consistent abnormalities on the day preceeding implantation. Additionally, by

means of ovarian transplants, he found that homozygous yellow embryos develop significantly further than normal in the uteri of non-yellow females, attaining greater size and forming Reichert's membrane and a rudimentary ectoplacental cone. Further histological analyses by Eaton and Green (1962, 1963) led them to postulate that the ultimate cause of death was failure to implant successfully, stemming from a markedly reduced degree of trophoblastic giant cell differentiation; it was proposed that subsequent malnutrition resulted from inability to establish an inverted yolk-sac placenta. This idea was supported by the observation that a small percentage of yellow homozygotes termed "escapers" (see Hadorn 1961) possess a natural capability of attaining a more advanced state of development, similar in degree to that described in the ovarian transplant experiments of Robertson, and that the stage ultimately reached before death is correlated with the number of giant cells formed. Eaton (1968) proceeded to demonstrate that the proportion of "escapers" could be significantly increased by injecting pregnant yellow mice with progesterone shortly before the implantation period, and thus speculated that subnormal levels of this steroid in heterozygote females might constitute an explanation of the prolonged survival of homozygotes in non-yellow mothers seen by Robertson. That conditions inside the uterus of yellow females may be generally suboptimal is also indicated by the finding that the viability of heterozygous yellow embryos appears to be lower in yellow mothers than in non-yellow mothers during the time between implantation and parturition (Wolff and Bartke 1965; 1966).

Recently Pederson (1974) has examined the development of A^y/A^y embryos *in vitro*. He was able to show that homozygotes are incapable of developing any further in culture than has been previously

observed to occur in utero, and then only after the artificial removal of the zona pellucida. The mutants were found typically to be capable of very limited trophoblastic outgrowth concurrent with complete degeneration of the inner cell mass; inter-strain crosses to promote hybrid vigour, failed to improve this performance. A notable element of this study was the detection of abnormality at the morula/early blastocyst stage, as judged by the presence of one or more arrested and excluded blastomeres. In a follow-up E.M. study, Calarco and Pederson (1976) were able to demonstrate that the ultra-structure of the excluded cells was consistent with arrest at the 8-cell stage, indicated by the rounded nucleoli, vacuolated mitochondria and numberous fibrous inclusions. They could find no evidence, however, of abnormality in the other (non-excluded) cells until degenerative changes had set in at a later stage. Abnormalities of fine structure, including immature-type mitochondria and vacuolated cytoplasm, have been observed in late blastocysts obtained from matings between yellow heterozygotes by Nowell and Chapman (1976). Unfortunately the very small number of embryos examined in this investigation can afford only tentative evidence that these abnormalities are specifically related to homozygosity for A^y.

The work presented here comprises both in vivo and in vitro techniques in an attempt to elucidate further the effect of the lethal gene with respect to interaction both between cells within the embryo, and between the embryo as a whole and its environment. A brief histological examination of the homozygotes in utero together with a study of their behaviour in culture (initiated prior to the publication of Pederson's work (1974)) serves to establish the lethality of the A^y allele

in the mouse stocks employed, and acts as a basis for interpreting further data. The viability of yellow homozygotes during delayed implantation, where trophoblast invasion of the uterus is not required for survival is investigated, and attempts are made to enhance development *in vitro* by supplementing culture medium with progesterone and with human cord serum. The degree of cell-autonomy expressed by the A^y allele with respect to its lethal action is also examined, by measuring trophoblastic outgrowth in cultured chimaeric embryos.

MATERIALS AND METHODS

Experimental Animals

The mice used in this study were obtained from the Institute of Animal Genetics, Edinburgh, and were derived from the C57 BL/6 strain into which the A^y allele had been introduced by repeated back-crossing. Adult yellow mice were consequently of the constitution A^y/a, since a (non-agouti black) is the standard allele at the agouti locus in this strain. a/a mice were thus regarded as wild-type for the purpose of this investigation, there being no evidence of developmental anomalies associated with this allele.

Matings between mice heterozygous for yellow (A^y/a x A^y/a) were used to produce litters of embryos containing the inviable homozygotes. Control litters were produced by mating a/a males with A^y/a females, or in the case of in vitro aggregation experiments by mating A^y/a males with a/a females. The latter procedure was adopted to permit maximum deployment of animal stocks, since it was regarded as unlikely that any differences in maternal environment could significantly influence the development of embryos cultured from the 8-cell stage. Mating cages were maintained in a fixed diurnal cycle. Copulation was determined by the standard vaginal plug method, and was assumed to occur during the middle of the dark period preceding the discovery of the plug.

Histological Procedures

(a) Standard Pregnancies

Females were sacrificed by cervical dislocation at $4\frac{1}{2}$ days (114 hrs.

post coitum). Uteri were fixed immediately in a mixture of 3 parts ethanol and 1 part glacial acetic acid for 12 hours at 4°C. Fixed uteri were dehydrated in ethanol, embedded in paraffin wax, and serially sectioned at 7µm. Mounted sections were then stained in Ehrlich's haematoxylin and eosin, or Azure B.

Cell numbers were estimated from serial sections as described in Appendix 1.

Additionally, a single litter of embryos from an A^y/a x A^y/a mating was flushed from the uterus at $3\frac{1}{2}$ days, fixed in gluteraldehyde followed by osmium tetroxide, and embedded in Epon according to the method of Luft (1961). 2µm sections were cut on an ultra-microtome and stained with Methylene Blue.

(b) Delay Pregnancies

Delayed implantation was induced artifically by means of the non-steroidal anti-oestrogen agent CI-628 which specifically inhibits the binding of oestradiol to cytosol receptors in the uterus (Katzenellenbogen and Ferguson 1975), and can be successfully employed in place of ovariectomy, to produce delayed implantation in rats (Callantine et al. 1966) and mice (N. Love, personal communication).

25 µ gms. of CI-628 (Parke-Davis, kindly donated by Dr. N. Love) was injected intra-peritoneally into pregnant mice at $2\frac{1}{2}$ days post coitum, and was followed-up by a subcutaneous dose of 1mg. progesterone (Depo-provera) on the morning of the next day to ensure maintenance of pregnancy.

Culture Techniques

(a) Single Embryos

Embryos were flushed from uteri late on the fourth day of pregnancy,

and washed twice in warm PB1 medium (Modified Dulbecco Phosphate Buffered Saline; Whittingham and Wales 1969). All embryos were artificially "hatched" prior to culture, by gently shearing off the zona pellucida with a slightly undersized micropipette, during the second wash. They were then transferred individually into pre-equilibrated 0.05ml.droplets of Eagle Minimal Essential Medium (Flow Labs) supplemented with 20% foetal calf serum (Flow Labs), 1.6mM glutamine and 100 units/ml.penicillin : pH was pre-adjusted to 7.3, and droplets were maintained under paraffin oil in Falcon plastic dishes at 36.5^{o}C in an atmosphere of 5% CO_2 in air. Medium was renewed after 48 hours of culture.

Embryos were scored individually for developmental state and morphological defects under a dissection microscope before zona removal, and during subsequent culture under phase-contrast optics with a Wild M40 inverted microscope. Development was recorded daily for a minimum of three days.

(b) Human Cord Serum

This experiment was performed as above, but with the substitution of heat-inactivated human cord serum for foetal calf serum, in the culture medium. Serum was prepared from fresh human placental blood obtained with the assistance of University College Hospital, London. The particular batch used had been employed successfully by other workers in the laboratory to enhance *in vitro* differentiation of post-implantation mouse embryos.

(c) Progesterone

This experiment was also performed as for standard culture,

but with the addition of 2 μ gms/ml crystalline progesterone (Wellcome) to the culture medium. This concentration was considered suitable since it was found by Whitten (1957) to be the maximum concentration which did not adversely affect the development of cleaving mouse embryos, and thus to represent a substantial increase above the levels present under standard culture conditions. The steroid was dispersed in the medium according to the method of Whitten, by dissolving it initially in glacial acetic acid. This solution made up to a concentration of 2 mgms./ml, was then added to the culture medium in the ratio of 1 μ litre/ml. The final pH of the medium, after equilibration in 5% CO_2 was readjusted to 7.3 by titration with 0.1N NaOH. In order to avoid possible diffusion of the steroid into paraffin oil during incubation, embryos were cultured in open dishes, rather than droplets, of medium.

(a) Culture of Aggregated Embryos

8-cell embryos, from experimental or control matings, were flushed from oviducts on the third day of pregnancy with PB1 medium, washed twice and transferred in pairs to 0.05ml droplets, under paraffin oil, of standard ova culture medium (Whittingham 1971). Zonae pellucidae were removed mechanically as described above. In order to maximise cell mixing during subsequent aggregation, naked embryos were firstly dissociated into single or paired blastomeres by rapid expulsion from a micropipette. Extreme care was taken to avoid rupturing the cells, and embryos seen to be partially damaged by this process were discarded. Total reaggregation was ensured by piling the blastomeres together at random in a small cavity, drilled

with the tip of a syringe needle into the plastic surface of the culture dish. After 24 hrs. incubation these were transferred as large aggregated morulae or blastocysts to droplets of outgrowth medium (Minimal Essential Medium + 20% foetal calf serum, as described above). In addition, a small group of single unaggregated embryos, from control matings, were cultured as a further control. These were treated in an identical fashion to the aggregated groups, including initial dissociation into individual blastomeres, but were allowed to develop individually. All embryos were cultured for a further 5 days after which time they were scored for trophoblast cell number under phase-contrast microscopy.

RESULTS

1. Histological Analysis

(Results are summarised in Tables 1 and 2)

$4\frac{1}{2}$ days p.c.

Seven uteri from A^y/a females mated to A^y/a males were sectioned at approximately 114 hours *post coitum*. A total of 55 embryos were found to be present, of which 42 appeared normal. Generally these were expanded blastocysts of about 65 cells (mean = 65.1 ± 1.9) which were undergoing the initial stages of implantation. A layer of primary endoderm could be seen covering the inner surface of the inner cell mass, and uterine attachment by the abembryonic polar trophoblast was firmly established. A number of incipient giant cells were normally present in the attachment region, and there was evidence of invasion of the uterine epithelium, which was starting to slough away in some cases. Limited decidual proliferation around the embryo could often be seen starting to seal off the lumen above and below the implantation site.

Thirteen embryos (23.6%), however, were quite clearly abnormal and exhibited peculiarities identical with those attributed to *yellow* homozygotes by earlier workers. Typically these were small collapsed or partially collapsed blastocysts of about 35 cells (mean = 33.5 ± 2.2). Cytoplasmic vacuolation was commonly visible and in some cases pycnotic or binucleate cells were also present. There did not appear to be a clearly delineated endodermal layer, although this was difficult to ascertain from the distorted morphology of the embryos. In the majority of cases there was some degree of attachment to the uterine

Plate 1 Embryos from A^y/a x A^y/a matings sectioned *in utero* at $4\frac{1}{2}$ days p.c.

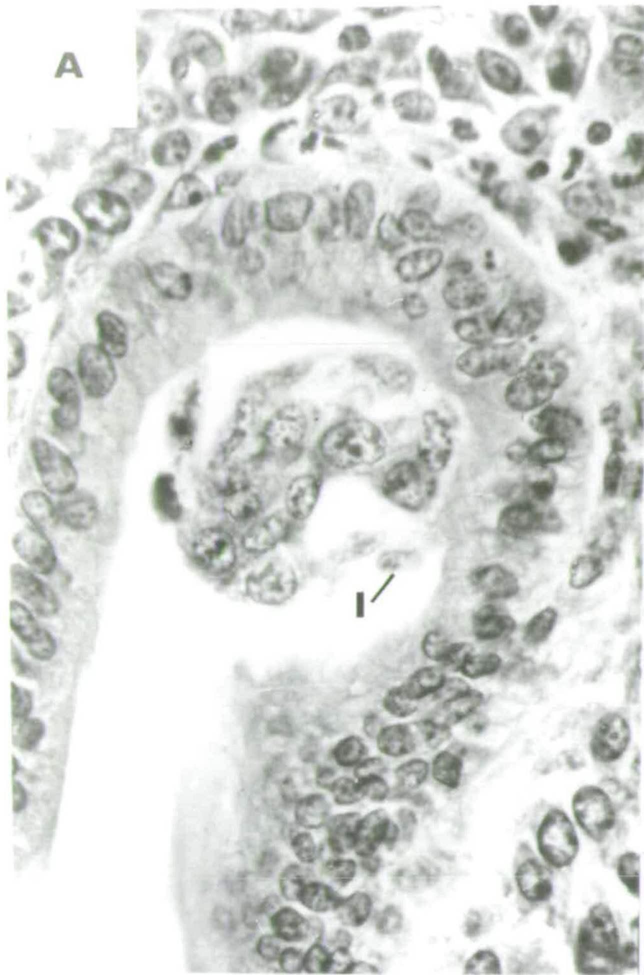
A Partially collapsed A^y/A^y blastocyst. Note primary invasive cell (I). Stained with H & E.

B Collapsed A^y/A^y blastocyst exhibiting invasive tendencies (I). Pycnotic (P) and fragmented (F) nuclei visible. Stained with H & E.

C A^y/A^y blastocyst with pycnotic nucleus (P). Note the intact and relatively untransformed uterine epithelium. Stained with Azure B.

D Normal blastocyst (littermate of B), with primary endoderm (E), undergoing the initial stages of implantation with primary invasive cells (I) penetrating the uterine epithelium. Stained with H & E.

(Magnification x 500)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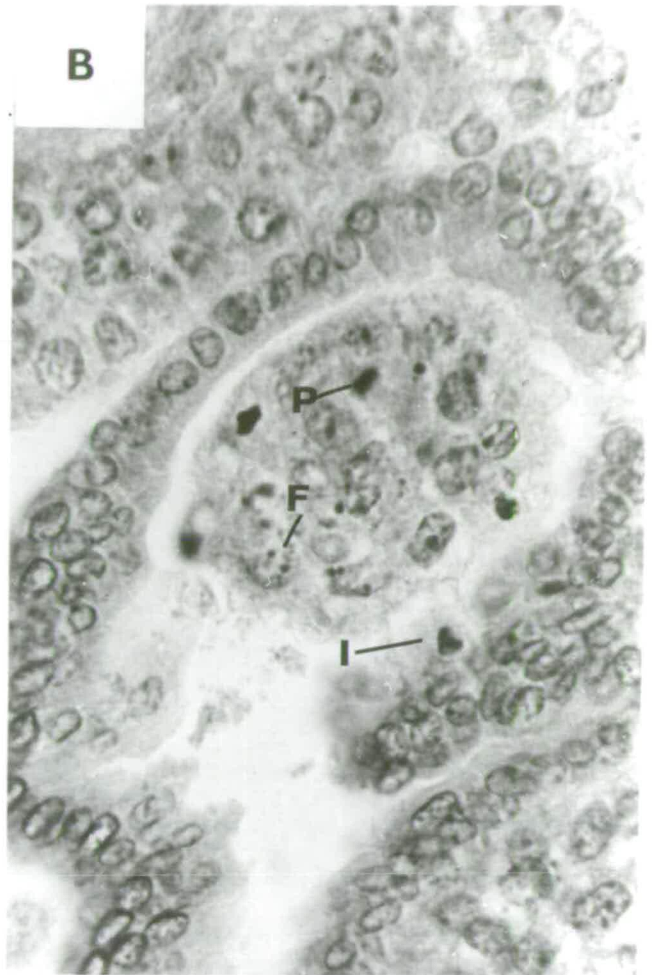
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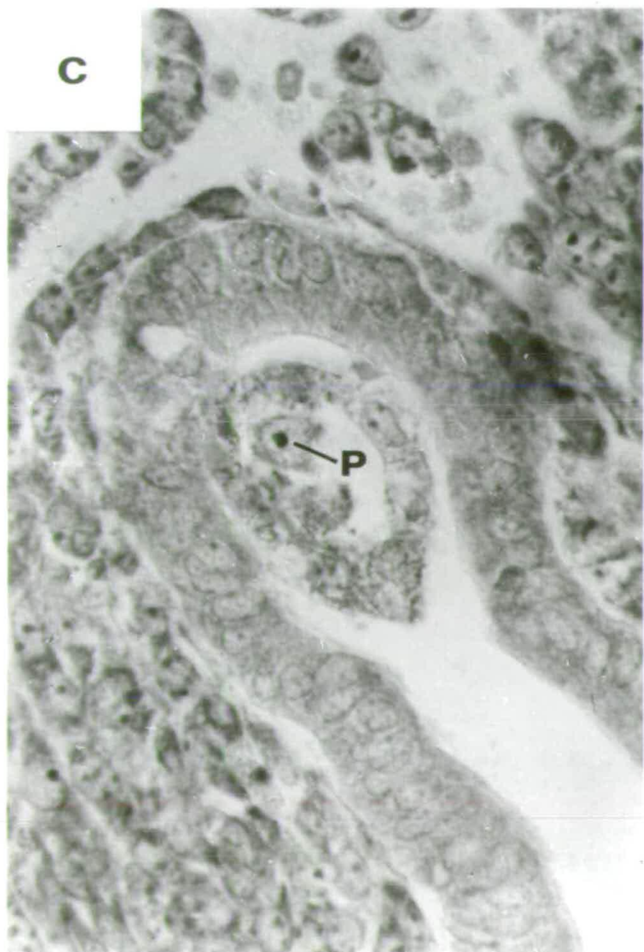
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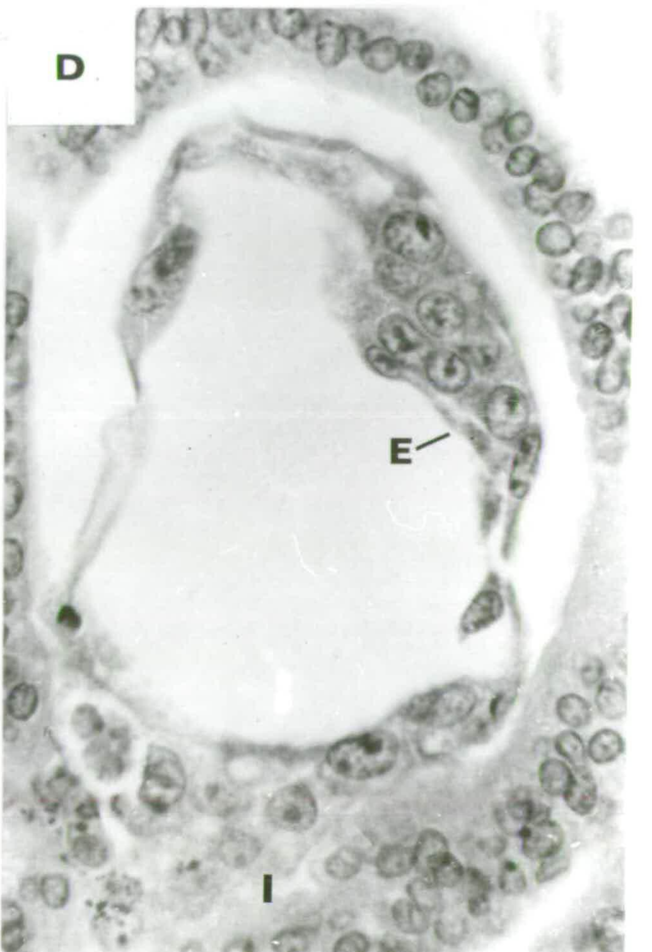
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epithelium although significantly more tenuous than normal, and in several of these one or two incipient giant cells were present, exhibiting distinctly invasive tendencies. The reaction of the uterus in the proximity of the abnormal embryos was in general similar to that seen with littermates, an initial degree of proliferation often being present. The epithelium, however, frequently seemed to be in a less advanced state of transformation and detachment compared with that of normal implantation sites. Sections stained with Azure B revealed no noticeable difference in intensity of uptake between normal and abnormal embryos, making it unlikely that any large discrepancies in RNA content existed (Flax and Himes 1953).

From the great similarity of the abnormal embryos to those described in the more detailed studies of other workers, and from their occurrence in numbers closely approximating to the Mendelian expectation of 25% , it was assumed that these represented yellow homozygotes ($X_1^2 = 0.05$).

$5\frac{1}{2}$ days p.c.

Five uteri from A^y/a females mated to A^y/a males, and sectioned at approximately 138 hours post coitum, contained a total of 34 decidual swellings. Twenty-five of these held normal egg-cylinder stage embryos, now substantially attached to the uterus over almost the entire surface of the trophectoderm, and surrounded on the lower lateral and anti-mesometrial margins by numerous (now clearly identifiable) trophoblastic giant cells.

Of the 9 (26.5%) remaining decidual swellings, 7 contained only scattered debris and maternal blood cells. In some cases a few necrescent

cells were identifiable as being of embryonic origin, but more often it was impossible to distinguish embryonic remains from the disintegrating remnants of the uterine epithelium. These were assumed to represent the abortive implantation sites of A^y/A^y embryos in accordance with the descriptions given by previous investigators.

Additionally, however, two swellings held somewhat degenerate blastocysts clearly distinct from their normal littermates by their marked developmental retardation, resembling more closely embryos of $4\frac{1}{2}$ days post coitum. Attachment, accompanied by a few giant cells, was consolidated at the antimesometrial pole only and the small inner cell mass, with scant covering of primary endoderm, had not yet started to enlarge downwards into the blastocoele. The genotype of these embryos could not readily be determined since they exhibited a more or less intermediate phenotype between mutant homozygote and littermate. It was noted, however, that these features are compatible with the "escapers" observed by Eaton & Green (1963), which represent a minority of yellow homozygotes capable of undergoing extended development for a limited period.

Delayed Implantation (See Table 2)

10 uteri from A^y/a females mated to A^y/a males, and fixed after 2 days of artificially induced delay, contained a total of 43 embryos. Of these, 34 displayed the normal elongated morphology of diapause blastocysts lying quiescent in luminal pockets, with the trophectoderm intimately apposed over most of its surface to the uterine epithelium. Mean cell number per embryo was estimated as 60.3 ± 2.6.

9 embryos (20.9%), however, were distinctly abnormal, possessing peculiarities very much like those of $4\frac{1}{2}$ day yellow homozygotes

Table 1

EMBRYOS IN SECTIONED UTERI FROM $A^y/a \times A^y/a$ MATINGS

Time p.c.	No. Uteri	Total No. Embryos	No. Abnormal Embryos	Mean Cell No. Normal Embryos	Mean Cell No. Abnormal Embryos
$4\frac{1}{2}$ days	7	55	13 (23.6%)	65.1 ± 1.9	33.5 ± 2.2
$5\frac{1}{2}$ days	5	34	9 (26.5%)	-	- *

* Only debris present in most cases.

Figures in parentheses represent % total no. embryos

No. of abnormal embryos at $4\frac{1}{2}$ days is not significatly different from 25% of total ($X^2 = 0.05$)

No. of abnormal embryos at $5\frac{1}{2}$ days is not significantly different from 25% of total ($X^2 = 0.04$)

All of these were small clusters of 25 or 30 cells (mean = 26.1 $\pm$ 1.3). Most were just recognisable as collapsed blastocysts, with vacuolated internal regions which appeared to represent the former position of the blastocoele, although trophoblast and inner cell mass could no longer easily be distinguished as separate entities. Several instances of binucleation were observed, and often vesiculating necrotic cells were present. Like their normal counterparts, they were almost totally surrounded by the uterine epithelium, however contact was only partial and extremely ragged.

By contrast, only 1 (3.8%) out of 26 embryos in 4 control uteri was abnormal, being totally necrotic.

After proportional correction from the control data for non-specific abnormalities, the X^2 test demonstrates that the number of abnormal embryos present in the experimental group is not significantly different from 25% (X^2 = 1.36). It is therefore concluded that the abnormal embryos were yellow homozygotes.

2. Culture of A^y/A^y Embryos over the Lethal Phase

Standard Culture Conditions

(Data summarised in Table 3)

From a total of 88 embryos flushed at approximately 90 hours *post coitum* from the uteri of A^y/a females mated to A^y/a males, 28 (31.8%) were classified prior to culture as being "abnormal". 17 of these exhibited no signs of cavitation and were consequently judged to be developmentally retarded, 5 possessed one or more excluded blastomeres of the type observed by Pederson, while the remaining 6 displayed both features.

Table 2

EMBRYOS SECTIONED IN UTERO AFTER 48 HOURS DELAYED IMPLANTATION

Mating	No. Uteri	Total No. Embryos	No. Abnormal Embryos	Mean Cell No. Normal Embryos	Mean Cell No. Abnormal Embryos
$A^y/a \times A^y/a$	10	43	9 (20.9%)	60.3 ± 2.6	26.1 ± 1.3
$A^y/a \times a/a$	4	26	1 (3.8%)	-	-

Figures in parentheses represent % total no. embryos

No. of abnormal embryos from $A^y/a \times A^y/a$ matings, after correction from control data is not significantly different from 25% of total ($X^2 = 1.36$)

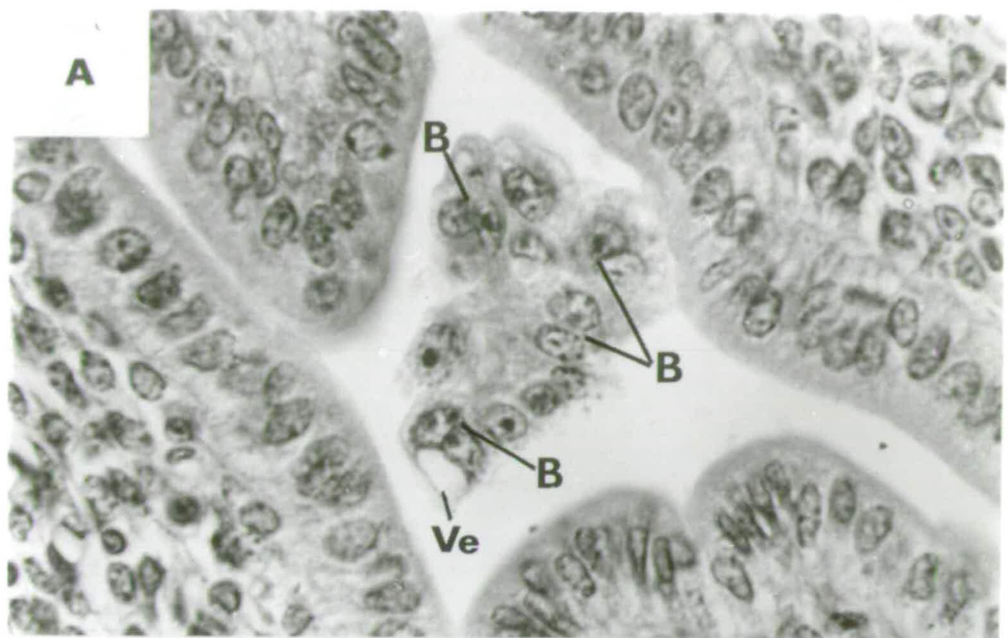
Plate 2 Embryos from A^y/a x A^y/a matings sectioned _in utero_ after 2 days delayed implantation.

A Presumptive A^y/A^y embryo containing several binucleate cells (B) and surface vesicle (Ve).

B Presumptive A^y/A^y embryo. Note the vacuolated central regions (V), and tenuous contact with the uterine epithelium.

C Normal delay blastocyst (littermate of B) displaying intimate contact between trophectoderm and uterine epithelium.

(Magnification x 500)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B
B
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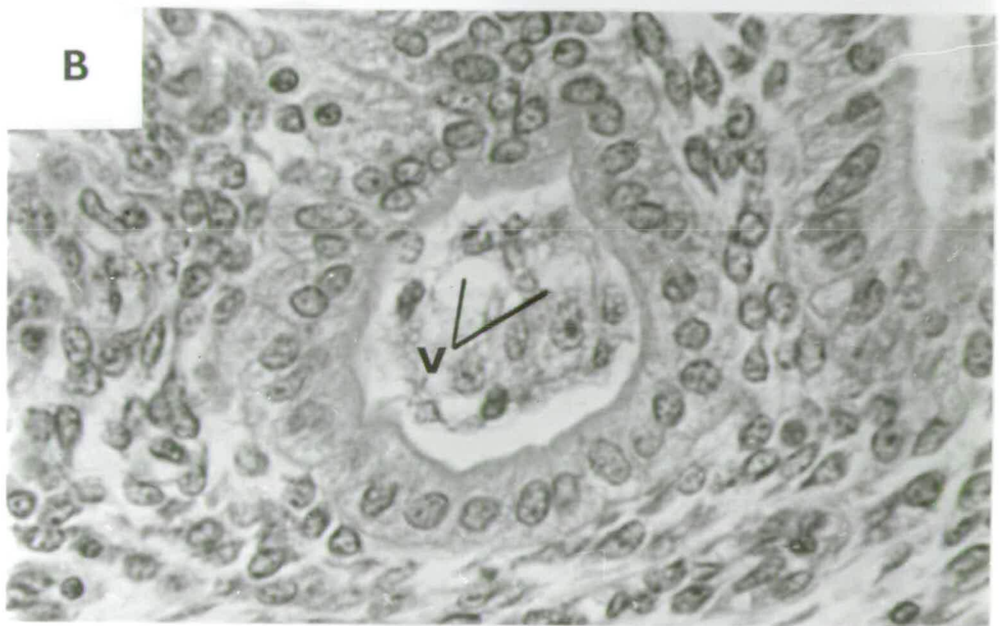
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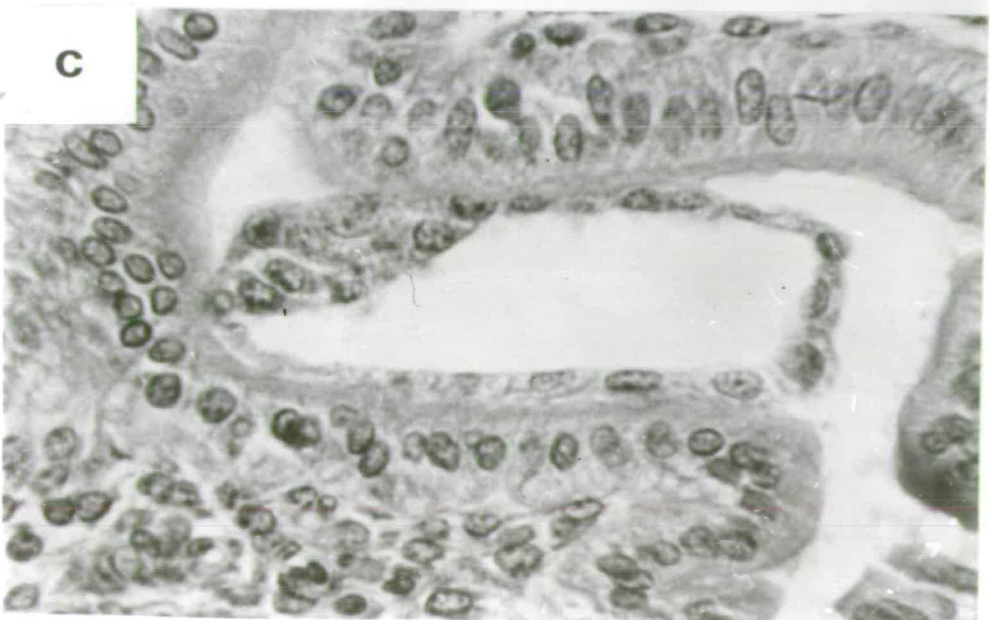

C

After 3 days of culture, 54 embryos (61.4%) had become attached to the surface of the culture dish and had undergone extensive trophoblastic outgrowth with enlargement of the inner cell mass. The remaining 34 embryos (38.6%) failed to undergo substantial development and had become degenerate by the end of the third day of culture. Often these successfuly attached to the culture dish, but subsequently degenerated into vesiculating balls of cells, extruding blunt pseudopodium-like cellular projections along the surface of the plastic before becoming totally necrotic. In a few cases a very limited degree of trophoblastic outgrowth was visible and persisted for one or two days after disintegration of the inner cell mass. Table 3 shows that 60.7% of those classified as "abnormal" before culture failed to outgrow normally compared with only 28.3% of those initially scored as "normal".

Of 54 control embryos from A^y/a females mated to a/a males, 5 (9.3%) were classified as abnormal before culture, comprising 3 uncavitated morulae and 2 blastocysts with excluded blastomeres. In all, 10 (18.5%) embryos failed to undergo normal outgrowth, forming small necrotic masses similar to those seen in the experimental group. These were composed of 60% of embryos originally classified as "abnormal" and 14.3% of those classified as "normal".

After correction for non-specific death from the control data, assuming that such mortality occurs equally in all genotypes, a net figure of 24.7% developmental failure is obtained for embryos from A^y/a x A^y/a matings. (See Appendix 2). From the excellent statistical agreement of this figure with the Mendelian expectation of 25%, it is concluded that in this system homozygous <u>yellow</u> embryos are unable to

Table 3

CULTURE OF $3\frac{1}{2}$ DAYS EMBRYOS FOR 3 DAYS IN STANDARD MEDIUM

Cross	Morphology Prior to Culture	No. Embryos	Normal Outgrowth	Little or Zero Outgrowth
A^y/a ♀ x A^y/a ♂	Normal Blastocyst	60	43 (71.7%)	17 (28.3%)
	Uncavitated	17	8 (47.1%)	9 (52.9%)
	Excluded Blastomeres	5	3 (60.0%)	2 (40.0%)
	Uncavitated plus Excluded Blastomeres	6	o (0.0%)	6 (100.0%)
	TOTAL	88	54 (61.4%)	34 (38.6%)
	Morphology Prior to Culture	**No. Embryos**	**Normal Outgrowth**	**Little or Zero Outgrowth**
A^y/a ♀ x a/a ♂	Normal Blastocyst	49	42 (85.7%)	7 (14.3%)
	Uncavitated	3	1 (33.3%)	2 (66.7%)
	Excluded Blastomeres	0	-	-
	Uncavitated plus Excluded Blastomeres	2	1 (50.0%)	1 (50.0%)
	TOTAL	54	44 (81.5%)	10 (18.5%)

Figures in parentheses represent percentages of numbers in left-hand column

Proportions of embryos outgrowing normally in experimental versus control groups are significantly different ($X^2 = 6.3 \quad p < 0.025$)

Proportions of initially abnormal embryos in experimental versus control groups are significantly different ($X^2 = 9.5 \quad p < 0.005$)

outgrow normally, producing at best a few trophoblastic giant cells and subsequently dying without further development.

The X^2 test indicates that there is also a highly significant difference in the proportion of embryos observed to be "abnormal" prior to culture between experimental and control groups ($X_1^2 = 9.55$, $p < 0.005$). This fact taken in conjunction with the observation that ~60% of all "abnormal" embryos in the experimental group were incapable of normal outgrowth, indicates a striking relationship between homozygosity for the A^y allele and abnormality on the 4th day <u>post coitum</u> , as judged in terms of retarded development, exclusion of blastomeres, or both.

<u>Human Cord Serum</u> (See Table 4)

Human cord serum has been shown to be markedly superior to commercially available foetal calf serum in facilitating the extended differentiation of mouse embryos <u>in vitro</u> (Hsu 1973; Hsu et al.1974) and has been found in this laboratory to noticeably enhance the vigour of inner cell mass development throughout post-implantation culture.

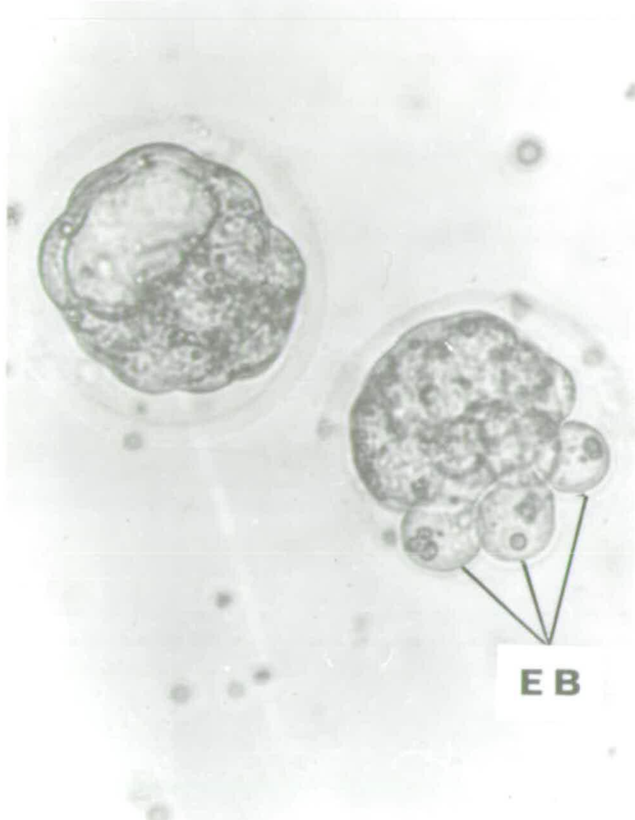
43 blastocysts from A^y/a x A^y/a matings were cultured under conditions identical to those of the previous experiment but with the substitution of human cord serum for foetal calf serum in the culture medium. 25 (58%) of these underwent substantial trophoblastic outgrowth, exhibiting prominent, well developed inner cell masses after 3 days of culture, with many continuing to develop up to the egg-cylinder stage. The remaining 18 embryos (42%), however, failed to outgrow and died shortly after attaching to the culture dish, showing no discernable improvement in development when compared to their counterparts cultured in foetal calf serum.

Plate 3 Abnormalities associated with A^y/A^y embryos flushed from the uterus at $3\frac{1}{2}$ days p.c.

A Morula with several excluded blastomeres (EB), alongside normal blastocyst (x 500)

B Blastocyst with excluded blastomere (EB), together with normal littermates (x 500)

C Epon embedded section (2μ) of blastocyst displaying large excluded (8 cell stage?) blastomere (x 1000). Stained with methylene blue.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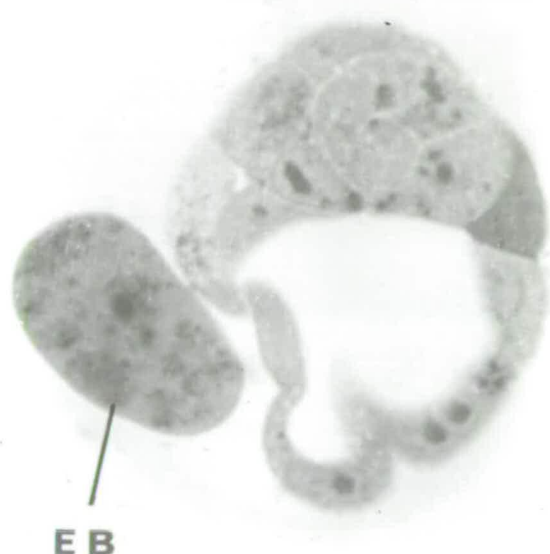
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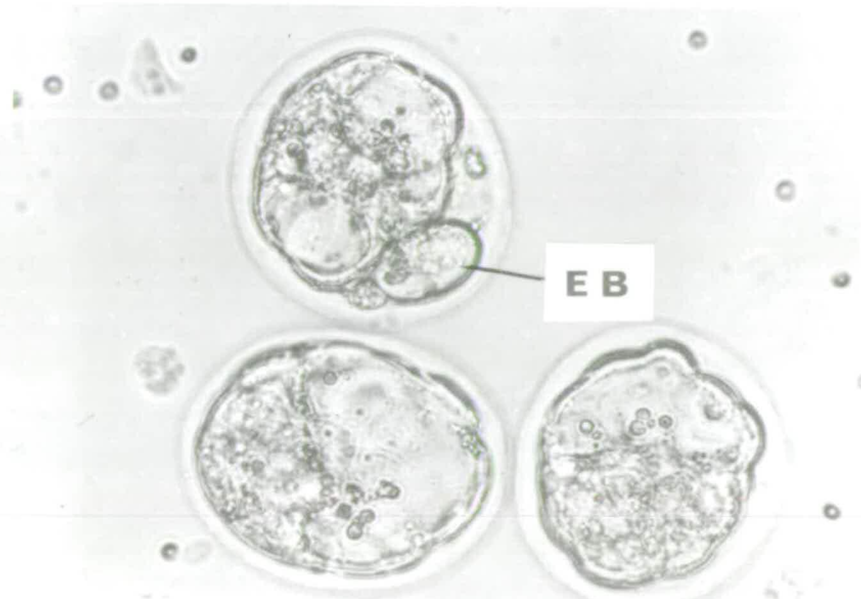
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Table 4

CULTURE OF 3½ DAY EMBRYOS FROM $A^y/a \times A^y/a$ MATINGS WITH DIFFERENT MEDIUM SUPPLEMENTS

Medium Supplement*	Total No. Embryos	Normal Outgrowth	Little or Zero Outgrowth
20% Foetal Calf Serum	88	54 (61.38%)	34 (38.62%)
20% Human Cord Serum	43	25 (58.14%)	18 (41.86%)
20% Foetal Calf Serum + 2μ gms/ml Progesterone	40	26 (65.00%)	14 (35.00%)

The figures in parentheses represent % of total no. of embryos

* Basic component in all groups was Eagle's Minimal Essential Medium

X^2 - test shows no significant heterogeneity between outgrowth ratios in the three treatments ($X^2_2 = 0.41$).

Since neither the percentage of embryos outgrowing, nor the degree of differentiation of those failing to outgrow normally, was substantially improved in this system, it is assumed that the superior conditions for development provided by human cord serum, do not enhance or extend the development of A^y/A^y embryos in culture.

Progesterone (See Table 4)

40 blastocysts from A^y/a x A^y/a matings were cultured in medium containing 2μ gms/ml progesterone. 26 (65%) embryos underwent normal extensive outgrowth. 14 (35%), however, failed to outgrow, in a manner identical to that previously described.

The X^2 test shows that the proportion of embryos which succeeded in outgrowing normally under these conditions is not significantly different from that observed using medium without progesterone (X^2 = 1.03). It is thus concluded that, at the concentration of progesterone employed, the performance of A^y/A^y embryos *in vitro* is not improved.

Culture of Aggregated Embryos

Two control groups were employed in this study, both containing embryos obtained from a/a females mated to A^y/a males, but composed of aggregated pairs and single embryos respectively. In each case all embryos were assumed to be genetically viable, the aggregated group consisting of the following combinations of genotypes:

$A^y/a \longleftrightarrow A^y/a$

$a/a \longleftrightarrow a/a$

$A^y/a \longleftrightarrow a/a$

In the absence of any evidence of difference in growth rate

Plate 4 Post-implantation culture of embryos from A^y/a x A^y/a matings.

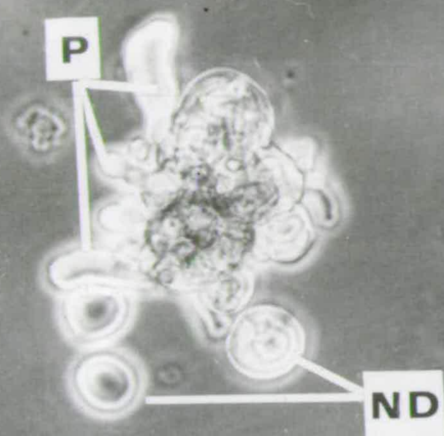
A & B Degenerating presumptive A^y/A^y embryos after 3 days of culture in standard outgrowth medium ($6\frac{1}{2}$ days p.c.). Note the pseudopodium-like projections (P) and necrotic debris (ND).

C Outgrowth from presumptive A^y/A^y embryo after 4 days of culture ($7\frac{1}{2}$ days p.c.) containing only two trophoblast cells. Part of the degenerate inner cell mass is still visible.

D Normal vigorous outgrowth from littermate of C after 4 days of culture ($7\frac{1}{2}$ days p.c.) with prominent inner cell mass (ICM) and prolific trophoblastic growth.

Phase-contrast illumination.

(Magnification x 300)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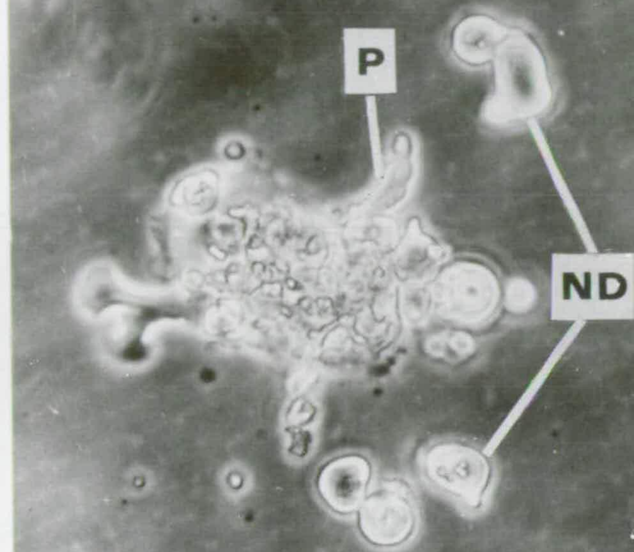
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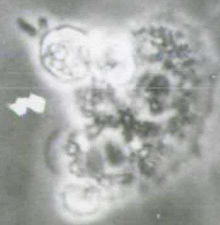
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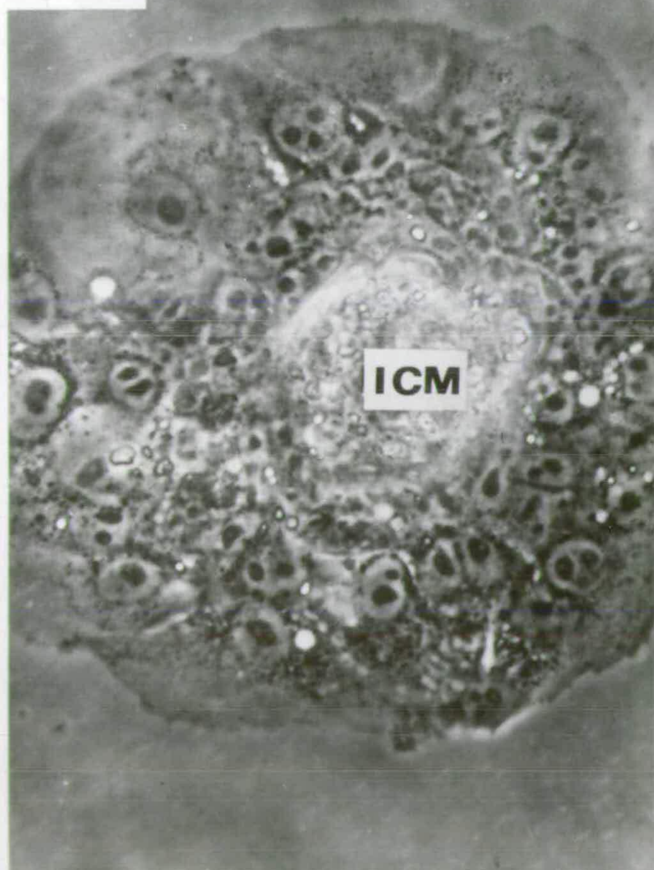
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ICM

between A^y/a and a/a embryos at this stage of development, all of these genotypic combinations were presumed also to possess the same developmental capacity.

The number of trophoblast cells in 41 aggregated control outgrowths and 17 single outgrowths treated in exactly the same fashion, were counted after 6 days of culture ($8\frac{1}{2}$ days post coitum). Excluding from the data 3 aggregates which died during culture, a mean value of 33.0 ($\pm$ 1.25) trophoblast cells per outgrowth was obtained for the aggregates, and of only 17.06 ($\pm$ 1.52) trophoblast cells per outgrowth for the singles. These figures establish that aggregated pairs of embryos will develop into outgrowths with almost twice the normal number of trophoblast cells.

Embryos obtained from A^y/a females mated to A^y/a males, and aggregated at random in pairs, by contrast may be expected to give rise to the following frequencies of genotypic combinations:

Category 1	$A^y/a \longleftrightarrow A^y/a$ $a/a \longleftrightarrow a/a$ $A^y/a \longleftrightarrow a/a$	56.25%
Category 2	$A^y/a \longleftrightarrow A^y/A^y$ $a/a \longleftrightarrow A^y/A^y$	37.5%
Category 3	$A^y/A^y \longleftrightarrow A^y/A^y$	6.25%

These expectations are calculated according to the binomial

$$p^2 + 2pq + q^2 = 1$$

where, p = combined frequency of A^y/a and a/a = 0.75

and q = frequency of A^y/A^y = 0.25

Category 1 comprises aggregates of totally viable genotype, as in the control group. Category 2, however, is composed of aggregates in which 50% of cells are homozygous for the yellow allele, and which

therefore may be partially or totally viable depending on the degree of cellular autonomy expressed by the lethal gene. Category 3 consists of embryos constructed totally of genetically inviable cells, and which are consequently unlikely to develop significantly further than their unaggregated homozygous equivalents.

Figure 1 shows the distribution, expressed as a frequency histogram of trophoblast cell numbers obtained for a group of 66 aggregates from $A^y/a \times A^y/a$ matings, together with that for the aggregated control group. It is at once evident that the distribution of the experimental group incorporates a class of outgrowths, in the region of 3-18 trophoblast cells per outgrowth, which is absent from the control group. This is confirmed statistically by the Kolmogorov-Smirnov test (see Campbell 1975) which enables a direct comparison to be made between histograms regardless of underlying distributions. The test demonstrates that there is a highly significant difference between the two distributions, and that the major area of discrepancy lies around the region corresponding to 15-18 trophoblast cells per outgrowth (Max. $D_S = 0.336$, $p \leq 0.01$).

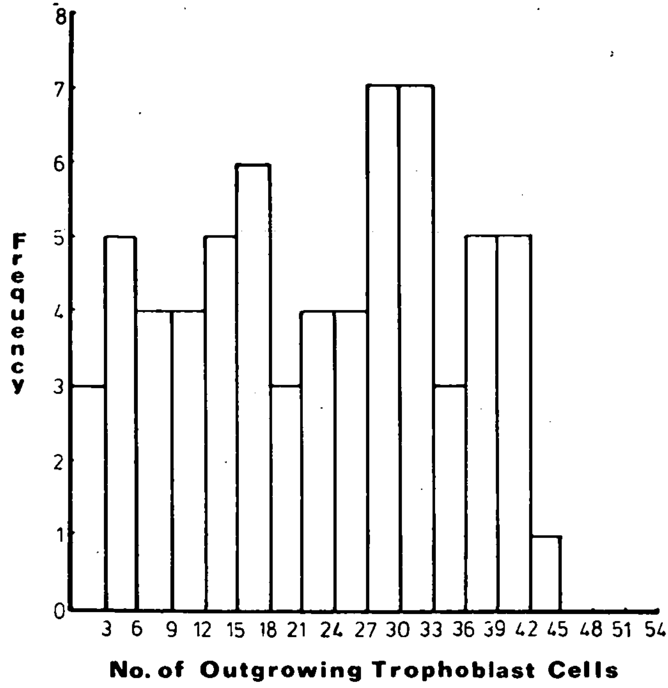
It is unlikely that a difference of this magnitude could be caused by the developmental failure of category 3 embryos alone, since only 4 such aggregates are expected on statistical grounds to be present in the entire experimental group. Almost certainly the source of the discrepancy is to be found in the behaviour of Category 2 aggregates, and it is of significance that the region of greatest difference between the two distributions coincides with mean value obtained for the single outgrowths. The data strongly indicate that cells of A^y/A^y genotype continue to be inviable even when aggregated with viable cells.

Figure 1 Distribution of trophoblast cell numbers in aggregate outgrowths, 6 days after aggregation at the 8-cell stage. ($8\frac{1}{2}$ days p.c.)

A Random aggregates between pairs of embryos from A^y/a x A^y/a matings (n = 66).

B Random aggregates between pairs of embryos from a/a x A^y/a matings (n = 41).

A



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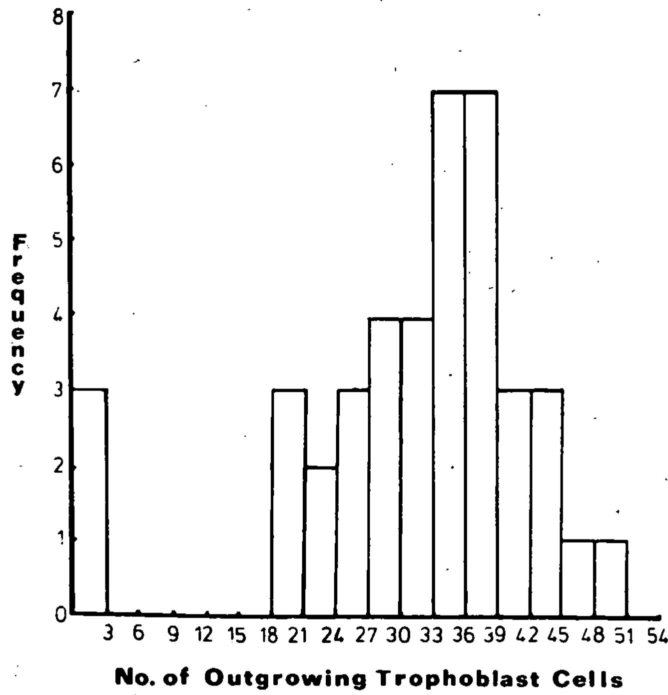
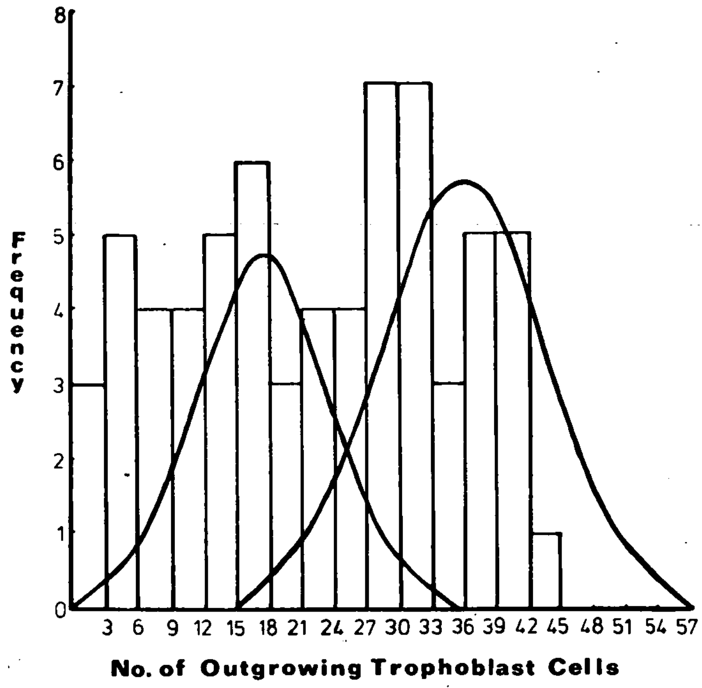


Figure 2

A Distribution of trophoblast cell numbers in aggregate outgrowths from A^y/a x A^y/a matings (as in Figure 1), overlaid by normal curves based on the means and standard deviations of aggregate and single control groups adjusted to represent 56.25% and 37.5% respectively of the total number of embryos (n = 66).

B Theoretical distribution of trophoblast cell numbers representing a group of 66 outgrowths composed of 56.25% viable aggregated embryos, 37.5% viable single embryos and 6.25% inviable embryos. Frequencies are based on the same normal curves as shown overlaid in A.

A



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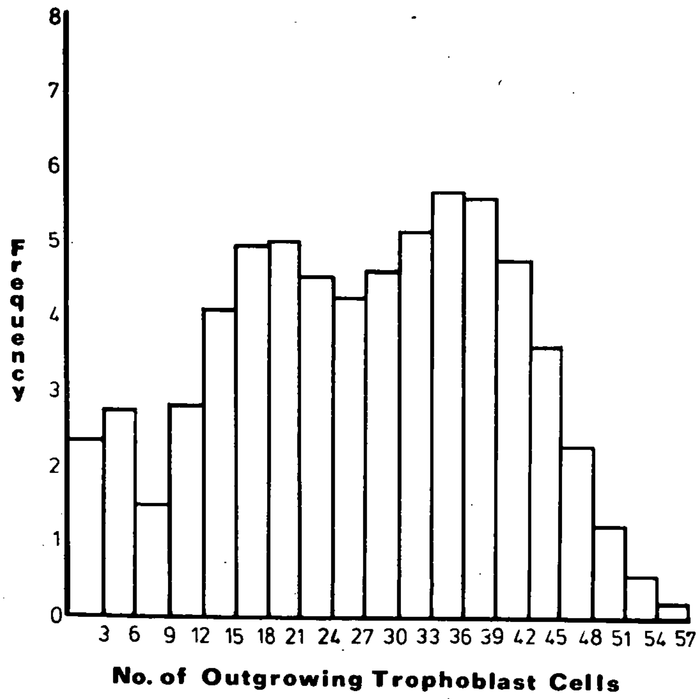


On this basis, only 50% of cells in Category 2 aggregates are competent to take part in normal post-implantation development, such that embryos with only a single complement of outgrowing trophoblast cells are formed.

This hypothesis may be tested by comparing the observed distribution of outgrowths in the experimental group with a theoretical distribution describing a population which is comprised of 56.25% aggregated outgrowths, 37.50% single outgrowths, and 6.25% inviable outgrowths. The frequency histogram of such a theoretical group, constructed from normal distributions of appropriate sizes based on the means and standard deviations of the two control groups, is shown compared with that of the experimental group, in Figure 2. 4.1 embryos (6.25%) are incorporated in the first two bars of the theoretical histogram, representing $A^y/A^y \longleftrightarrow A^y/A^y$ aggregates capable of little or no outgrowth. The normal distributions from which the theoretical histogram is compounded are shown overlaid on the observed histogram for further comparison. The Kolmogorov - Smirnov test reveals no significant difference between the distribution of frequencies in the two histograms (Max. $D_S = 0.152$).

It is therefore concluded that the A^y allele is autonomous with respect to cell lethality in this system.

DISCUSSION

It is clear from the histological evidence that the majority of homozygous yellow embryos fail to undergo the primary stages of implantation in a normal fashion, and subsequently degenerate before being rapidly resorbed. This is in general agreement with accounts of previous investigators, which support one another with respect to the effective lethal phase, although they differ somewhat regarding details of the local uterine response.

The interpretation of the events bringing about the death of the embryo however, as proposed by the authors of the most rigorous histological study (Eaton & Green 1962, 1963), no longer seems tenable both as a result of the investigations of Pederson and of the data presented here. As stated earlier, it was concluded by reconstructing the behaviour of embryos from the picture presented in fixed specimens, that yellow homozygotes perish from malnutrition as a direct result of their failure to implant normally (Eaton & Green 1963). Attention was consequently focused on the insufficiency of trophoblastic giant cell transformation as the root cause of developmental decline in the mutants.

Theories based on such a causal relationship must be re-examined in the light of certain experimental observations. Firstly, a high proportion of yellow homozygotes show signs of partial arrest or developmental retardation when flushed from the uterus one entire day before primary implantation is due to occur. Indeed, as noted by Pederson (1974) often the size of the excluded blastomeres exhibited by these embryos indicates their arrest at the four to eight cell stage. Whilst such abnormalities are not unique to the experimental group of

embryos in the present study, they show a strong statistical association with presumed yellow homozygotes. Kirkham (1917, 1919), in the first histological investigations, also suspected a link between pre-implantation abnormalities and homozygosity for yellow, recording the presence of abnormal morulae and of shrunken "blastulas" containing crowded cells and an abnormally small "blastodermic vesicle". Secondly, evidence from sectioned material in the present study demonstrates that the mutant embryos, at the time of primary implantation of their littermates, contain only about half the normal number of cells. This indicates that division either had been arrested totally during the previous cell cycle, or had been partially disrupted from an even earlier stage. Arrest and exclusion of one or more blastomeres during early cleavage might also be expected to contribute to a reduced cell number.

These observations are supported by the fact that homozygous yellow embryos exhibit an almost identically abnormal morphology during delay, although implantation is not required for survival and cell division is limited. Again cell number is very low, and typical symptoms of morbidity such as vacuolation and binucleation are evident.

Taken together, these findings constitute sufficient evidence to indicate that embryos homozygous for the A^y gene are developmentally disadvantaged prior to the implantation stage.

The more specific postulation that insufficient differentiation of trophoblastic giant cells is the major factor in developmental arrest seems unlikely in view of the observation that normal development of the inner cell mass in the mutant embryos does not occur *in vitro*

even under the optimal conditions for differentiation of this structure provided by human cord serum. Further it has been shown that both trophoblast and inner cell mass, when cultured in isolation from one another are capable of growth and differentiation (Ansell and Snow 1975; Solter and Knowles 1975). It is therefore difficult to account for the degeneration of the inner cell mass of homozygous yellow embryos in vitro (also observed by Pederson), other than by the assumption that the mutant gene renders this tissue inviable also. In particular it is striking that in the investigation of Pederson, and to a lesser extent in the present study, the only cells seen to develop when cultured over the lethal phase were a few trophoblastic giant cells.

Dickson (1967) has shown that progesterone administered on the 4th day of gestation is required for trophoblastic giant cell transformation in normal ovariectomised mice. Pursuing this fact, Eaton (1968) demonstrated that a 10-fold increase in the number of A^y/A^y embryos undergoing extended development in vivo (escapers) could be obtained by injecting yellow females with optimal doses of progesterone prior to implantation. It was thus tentatively suggested that heterozygous yellow females may be deficient in progesterone and that the uterine environment is consequently suboptimal. The fact that progesterone failed to enhance the development of A^y/A^y embryos in vitro is unhappily inconclusive, since it is not clear whether the hormone exerts its effect in vivo directly on the embryo or indirectly via the uterus. Dickson stresses that progesterone is only one factor affecting implantation and that a synergistic interaction with oestrogen is probably involved. Addition of progesterone alone to the culture medium may

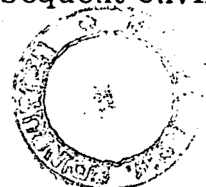
therefore be inadequate to elicit an observable response. Since Robertson (1942) found that A^y/A^y embryos permitted to develop in the uteri of non-yellow females, by means of ovary transplantation, underwent extended development similar to that produced by Eaton with injections of progesterone, it seems likely that the effect of both procedures is simply to optimise conditions for development such that the ailing mutant embryos are capable of surviving marginally longer. The argument is strengthened by Dickson's additional observations that progesterone (in utero) is not only required for trophoblastic giant cell transformation, but also causes blastocyst elongation and growth of the inner cell mass. An alternative theory, forwarded by Eaton & Green (1963), which might also explain the failure of progesterone to act on A^y/A^y embryos in vitro, is that the high concentrations of the steroid optimise conditions for the retarded homozygotes in vivo merely by slowing down the gestational development of the uterus and thereby decelerating the rate at which the two grow out of phase with one another. This idea is, in turn, supported by the fact that Eaton's mice were not ovariectomised prior to the injections of progesterone; since there is no direct evidence for low levels of the substance in A^y/a females it is possible that the resultant systemic concentration was greatly above normal in his experiments.

It may be concluded that almost certainly the effect of the mutant gene is not tissue specific, but interferes with the metabolism of the entire embryo at a stage in development which merely precedes or coincides with trophoblastic giant cell transformation.

The aggregation experiments appear to indicate partial or

complete autonomy of the A^y allele with respect to recessive lethality in this system. It should also be noted that while there is no "rescue" of mutant cells by viable cells, neither is there evidence of lethality spreading in the reverse direction into genetically viable cells. A similar experiment was performed by Mintz (1964 c) who attempted to show autoradiographically that cells of embryos homozygous for the t^{12} lethal factor remained inviable when aggregated with wild-type as judged by uptake and incorporation of $[^3H]$ - uridine. Unfortunately the lack of cell mingling which seems to be inherent in the standard method of aggregation employed (Garner & McLaren 1974), possibly reduced further by the nature of the mutant cells themselves, is coincidentally demonstrated by Mintz's autoradiographs and thus renders the result less than totally conclusive.

Indeed, care is always required when interpreting results of this kind which are essentially of a negative nature, especially where the function of the gene is entirely unknown. Almost certainly the lethal cells behave autonomously with respect to the system employed here, however the tacit assumption is made that the period at which critical gene function takes place is not greatly removed from the effective lethal phase expressed in the mutant embryos. As discussed above, there is substantial evidence to show that <u>yellow</u> homozygotes are abnormal prior to the lethal phase, possibly at the 8-cell stage or even earlier. This being so, the events which ultimately cause the death of the embryo and which may, or may not, be subject to modification by outside influences, could take place before aggregation such that the cells are destined to die irrespective of subsequent environmental



conditions. Unfortunately it was not considered feasible to test this possibility due to the poor post-implantation performance of embryos from this strain cultured continuously from the 2-cell stage. In addition, only outgrowing trophoblast cells were examined in this experiment since the extremely variable 3-dimensional structure of the inner cell mass is not generally amenable to microscopic measurement in situ. Consequently it must be noted that autonomy has been demonstrated for trophoblast only, and the viability of homozygous yellow inner-cell-mass cells in aggregates is yet undetermined.

The biochemical basis of A^y gene action remains a mystery. There is now no reason to believe that the gene is tissue specific; also the observations of early abnormality in conjunction with the capability of some embryos to undergo extended development in utero, especially under optimised conditions, are indicative of death resulting from a gradual degenerative process rather than from inability to respond to a stage-specific developmental requirement.

SECTION 3

A STUDY OF EMBRYOS HOMOZYGOUS FOR OLIGOSYNDACTYLISM (Os)

INTRODUCTION

The mutation oligosyndactylism (Os) causes fusion of the 2nd and 3rd digits in all four feet of mice heterozygous for the allele (Gruneberg 1956, 1961). Anomalies of muscular anatomy in the limbs (Kadam 1962) and a form of diabetes insipidus (Falconer et al.1964) also usually accompany the mutation, with high renal output and inability to concentrate urine arising from a paucity of nephrons in the kidneys (Stewart & Stewart 1969).

No satisfactory explanation, however, has been advanced to link these effects with the embryonic lethality which Os causes in the homozygous condition and which is the subject of this study. The time of death of Os/Os embryos *in utero*, has been determined in an histological investigation by Van Valen (1966), who found that the phenocritical phase occurs on the 4th day *post coitum*, leading to death during the following 24 hours with subsequent resorption. Affected blastocysts were found to possess increasing numbers of abnormally pale-staining cells, lacking a nuclear membrane and nucleolus, and in which the chromatin lay scattered and fragmented in the cytoplasm. The frequent occurrence of these cells in pairs, together with the general resemblance of the chromatin to mitotic figures, led the author to postulate that the mutation interferes with cell division. It was suggested that cells undergoing the 7th or 8th cleavage are either unable to complete division or cannot successfuly re-enter interphase with resultant cytostasis and developmental arrest.

Similar recessive mutations which disrupt cell division are the *lethal mitotic* (l m) factor in the urodele Pleurodeles waltlii (Gallien & Collenot 1964; Gallien 1974) and *lethal polyploid* (lpl)

in Drosophila hydei (Gloor 1951; Staiger and Gloor 1952). The critical phase of both mutations, however, occurs at a stage considerably later in development than that of Os, the former causing progressive blockage of mitosis in tail-bud stage larvae, and the latter producing polyploidy in the cells of the imaginal discs shortly before pupation. Unfortunately, as yet it is extremely difficult to assess the degree of analogy between Os and these mutations, since the patterns and stages of development are so different in the respective animals involved. Ultimately the similarities of phenotypic expression may prove to be largely superficial.

The purpose of this study was to confirm Van Valen's findings and to examine in more detail the nature of the abnormalities described, with a view to formulating a basic hypothesis regarding the lethal action of the Os allele. To this end, the results of an histological study of Os/Os embryos in normal pregnancy and lactational delay, together with an examination of chromosome morphology in air-dried spreads of the affected cells, are presented here. These are further supported by observations of the behaviour of the mutant embryos when cultured over the lethal phase.

MATERIALS AND METHODS

Experimental Animals

The adult mice used in this study were F1 hybrids obtained from matings between males heterozygous for oligosyndactylism (Os / +) derived from linkage stocks at the Institute of Animal Genetics, Edinburgh and wild-type females (+/ +) of the highly fertile random bred CD-1 strain (Charles River Ltd). This cross was adopted to circumvent breeding difficulties experienced with the original line, and also proved an efficient method of rapidly producing stocks of experimental animals.

Matings between F1 mice heterozygous for the Os allele (Os / + x Os / +) were employed to produce litters containing homozygous mutant embryos. Control litters were obtained by mating wild-type males with heterozygous females (Os / + x +/ +), thereby ensuring that any unforeseen effects of the mutation on the maternal reproductive system or on the cytoplasm of the ova would be equalised in both groups. Females, aged 8-16 weeks, were caged in groups of three or four with single males, and copulation determined by the presence of a vaginal plug. To obtain embryos in lactational delay, single females in the final stages of pregnancy were caged with males and checked for post-partum mating 24 hours after the birth of the litter.

For convenience of timing, animals were maintained in an artificially reversed diurnal light cycle. Mating was assumed to occur during the middle of the dark period.

Histological Procedures

(a) Females were killed by cervical dislocation at 5 days (120-122 hrs)

or 6 days (144-148 hrs) _post coitum_ for standard pregnancies, and at $6\frac{1}{2}$ days (163-164 hrs) _post coitum_ for lactational delay pregnancies. Uteri were excised, and fixed in a mixture of 3 parts ethanol and 1 part glacial acetic acid for 12 hrs at $4^{o}C$. Specimens were subsequently dehydrated, and embedded in paraffin wax. Serial sections were cut at 6µm, stained in haematoxolin and eosin, and mounted in DPX. Cell numbers were estimated from serial sections as described in Appendix 1.

(b) Air-dried spreads of whole embryos were prepared according to an adaptation of the method described by Evans et al.(1972). Blastocysts were flushed from uteri at approximately 115 hours _post coitum_, this being the latest time at which reasonably intact embryos could be obtained, although yield was often low. These were incubated for 15 minutes at $37^{o}C$ in hypotonic solution (0.8% sodium tri-citrate in H_2O), followed by a 30 minute fixation in 3 parts ethanol and 1 part glacial acetic acid. Each embryo was then transferred in a minimum volume of fixative onto a clear microscope slide, and a small drop of 60% acetic acid added immediately. As soon as the embryo had started to disaggregate, the drop was drawn across the slide with the tip of a micropipette, leaving a trail of ruptured cells adhering to the surface. Preparations were allowed to dry for 30 minutes at room temperature before staining with Geimsa or Acetic-orcein.

Culture

Blastocysts at approximately 95 hours _post coitum_ were flushed from uteri by syringe with PB1 medium (Whittingham & Wales 1969)

into an embryological watchglass. Zonae pellucidae were removed artifically at this stage by gently shearing with a small-bore micropipette. After two washes in flushing medium, embryos were transferred singly into 0.05ml droplets of Eagle Minimal Essential Medium (Flow Labs) supplemented with 20% foetal calf serum (Flow Labs.), 1.6m M glutamine and 100 units/ml. penicillin. pH was pre-adjusted to 7.3, and droplets were maintained under paraffin oil in Falcon plastic culture dishes at 36.5^{o}C in an atmosphere of 5% CO_2 in air. Medium was renewed after 48 hours of culture.

Progress of individual embryos was recorded daily following examination, w ith a Wild M40 inverted microscope, under phase contrast illumination. After 72 hours of culture, the number of outgrowing trophoblast cells in each embryo was determined, and the area of outgrowth measured with a calibrated micrometer grid.

RESULTS

Histological Analysis

5 days p.c.

(Data summarised in Table 1)

A total of 45 embryos in 6 uteri from Os/+ x Os/+ matings were sectioned at this stage. The majority were present as late blastocysts/ early egg cylinders with the inner cell mass covered on its blastocoelic surface by a well defined layer of endoderm, and bulging downwards into the cavity. The uterine epithelium was becoming detached from the underlying stroma in the crypts surrounding the embryos, and distinct signs of penetration and attachment by the antimesometrial and lateral trophoblast were evident, indicating that implantation was under way.

12 embryos (26.7%) , however, were quite clearly abnormal. These were presumed to be Os/Os on account of both the overwhelming similarity to the mutant homozygotes previously described by Van Valen (1966), and of the excellent statistical agreement with the Mendelian expectation of 25%. Gross morphology was usually somewhat misshapen, and frequently the blastocoele had collapsed such that the trophectoderm was folded up around the inner cell mass. Nevertheless, in most cases signs of attachment and initial implantation could clearly be seen. On average approximately 40% of cells in these embryos were in a state resembling metaphase and they were consequently quite distinct from their littermates, which typically displayed only one tenth of this number of metaphases (4%). Cells of this type were particularly frequent in the inner cell mass, but could be seen in all regions of the embryos, although sometimes conformation had become so distorted

Table 1

5-DAY EMBRYOS SECTIONED IN UTERO FROM Os/+ x Os/+ MATINGS

Class of Embryos	No. of Embryos	%of Total Embryos	Mean No. Abnormal/ Mitotic Cells	Mean Total Cell No.	Mitotic Index
Presumptive Os/Os	12	26.7%	38.0 ($\pm$ 2.8)	96 ($\pm$ 9.5)	39.6%
Littermates	33	73.3%	5.6 ($\pm$ 0.7)	130 ($\pm$ 6.7)	4.2%

No. of presumptive Os/Os embryos is not significantly different from 25% of total ($X^2 = 0.24$)

Mean total cell number is significantly different in the two classes ($t = 2.9$, $p < 0.01$)

Plate 1 Embryos from Os/ + x Os/+ matings sectioned *in utero* at 5 days p.c.

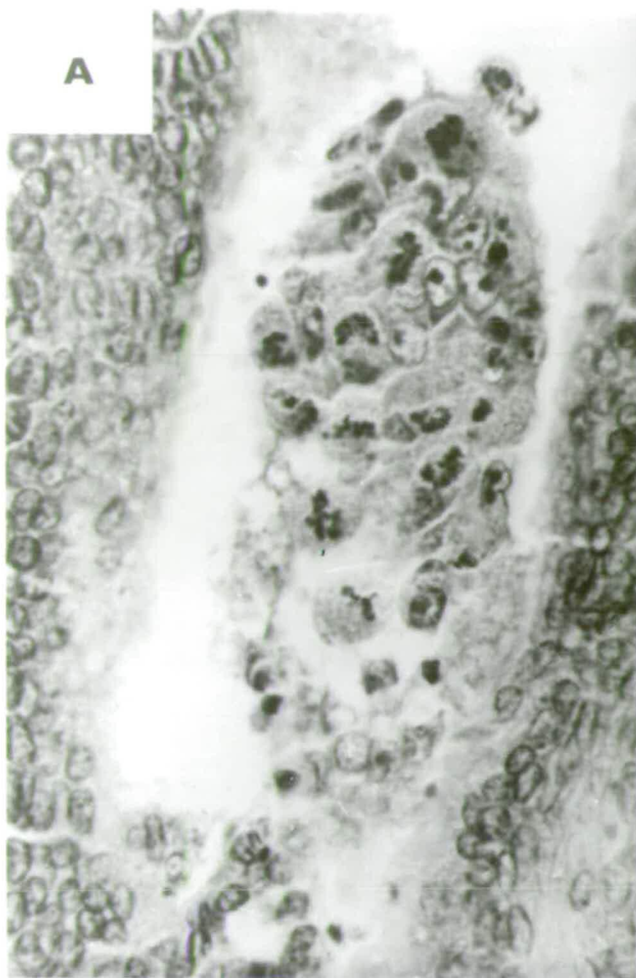
A Os/Os embryo displaying numerous mitotic/abnormal cells. Attachment to the uterus via the polar trophoblast is evident.

B Section through collapsed Os/Os embryo.

C Section through folded-up trophoblast of collapsed Os/Os embryo. Uterine attachment with local erosion of the epithelium can be seen.

D Normal littermate of A, displaying only one mitotic cell (M). The uterine epithelium has been totally eroded and an invading trophoblastic giant cell (T) can clearly be seen.

(Magnification x 500)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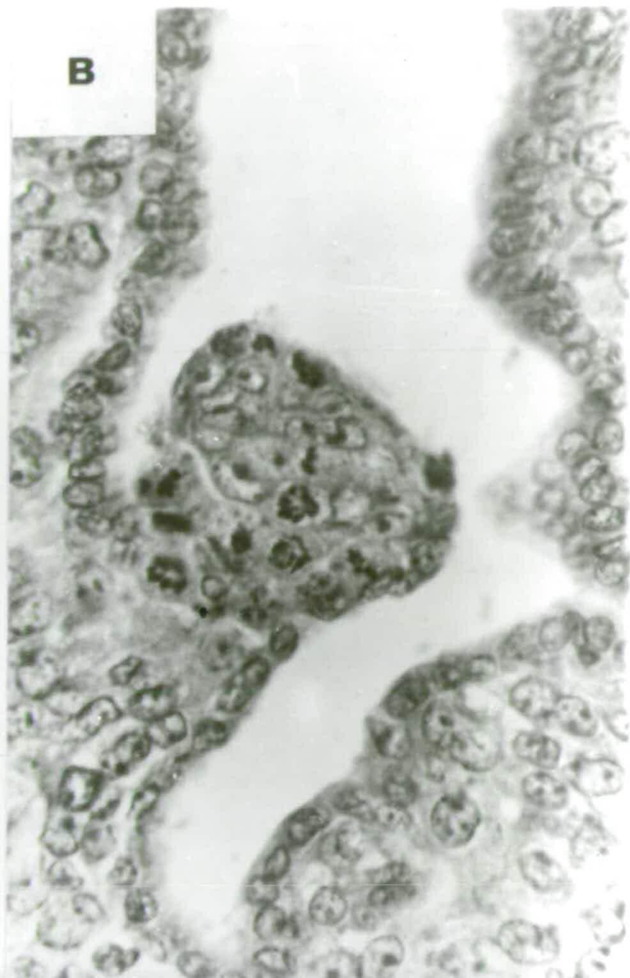
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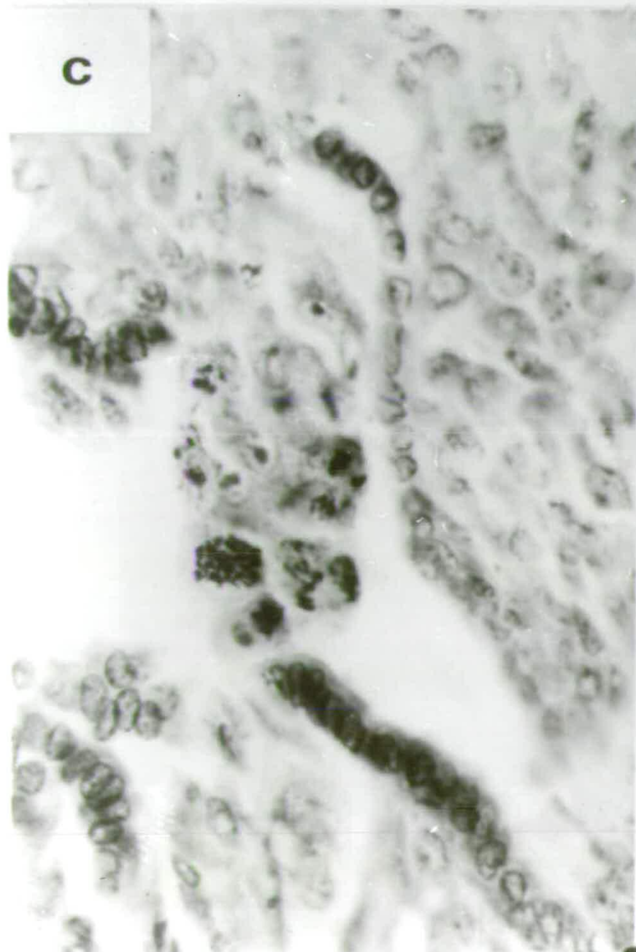
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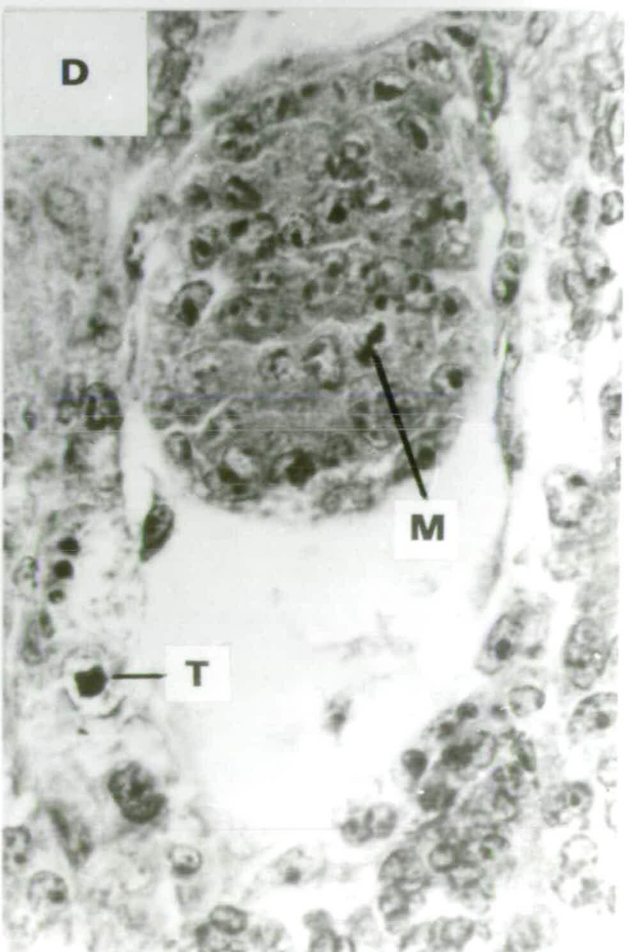
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M
T

that it proved difficult to distinguish between different tissues. In some of these cells the arrangement of the chromatin was sufficiently well ordered to be identical to that of normal metaphase; in others, the chromatin was fragmented and scattered haphazardly throughout the cytoplasm. In addition, the affected cells were usually large, rounded and pale-staining, without trace of nuclear membrane or nucleolus. The appearance of the other (nucleated) cells in the embryos was quite normal.

Cell number was estimated according to Abercrombie's formula (see Appendix 1) for nucleated cells, and by direct counting for mitotic/abnormal cells since the irregular size and distribution of the chromatin did not lend itself to the former method. The mean number of mitotic/abnormal cells per embryo was thus calculated as 38.0 ($\pm$ 2.8) for Os homozygotes and as 5.6 ($\pm$ 0.7) for littermates, while mean total cell number per embryo was estimated at 96 ($\pm$ 9.5) and 130 ($\pm$ 6.7) for each group respectively.

Students' t-test confirms that cell number in the Os homozygotes is significantly lower than in littermates ($p < 0.01$).

<u>6 days p.c.</u>

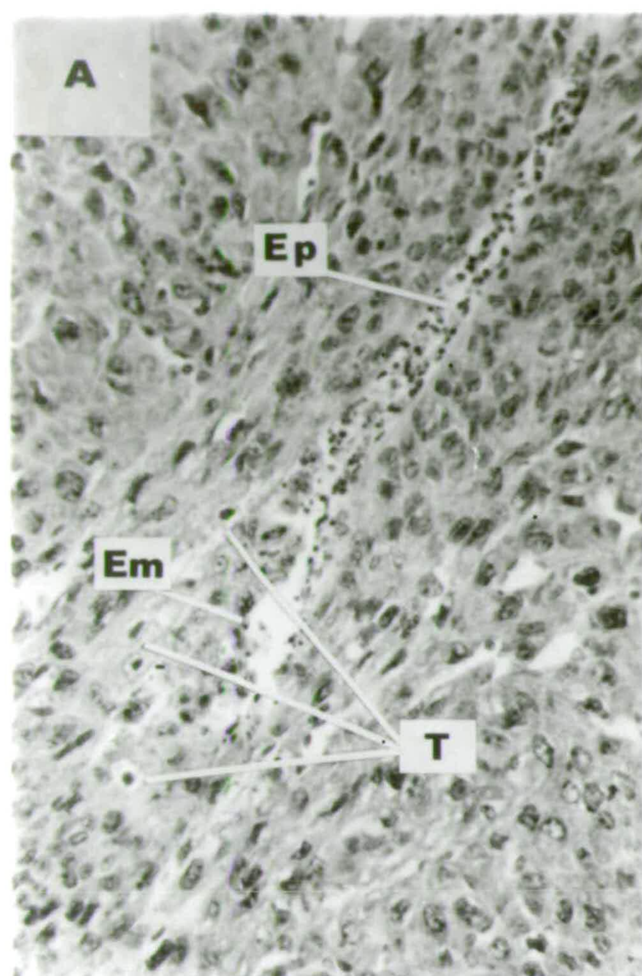
Normal embryos at this time were well formed egg-cylinders. Attachment was now complete, and each implantation site was surrounded by a marked decidual swelling. A noticeable feature was the presence of large invasive trophoblastic giant cells both in the mural trophoblast of the embryo, and buried deep within the surrounding uterine tissue. These cells were quite distinctive on account of their large nuclei and nucleoli, and their pale elongated cytoplasm penetrating between neighbouring decidual cells.

Plate 2 Remnants of Os/Os embryos sectioned *in utero* at 6 days p.c.

A Degenerate remains of Os/Os embryo (EM). Apparently normal trophoblastic giant cells (T) have invaded the decidual tissue around the site. Remnants of the uterine epithelium (Ep) are still present (x 175).

B Trophoblastic giant cells (T) at the site of a degenerating Os/Os embryo. Three cells are normal in all respects, although one other appears to contain some fragmented chromatin of unknown origin (x 175).

C Enlargement of embryonic remains from A, showing in more detail the giant cells together with scattered mitotic/abnormal cells. (x 500)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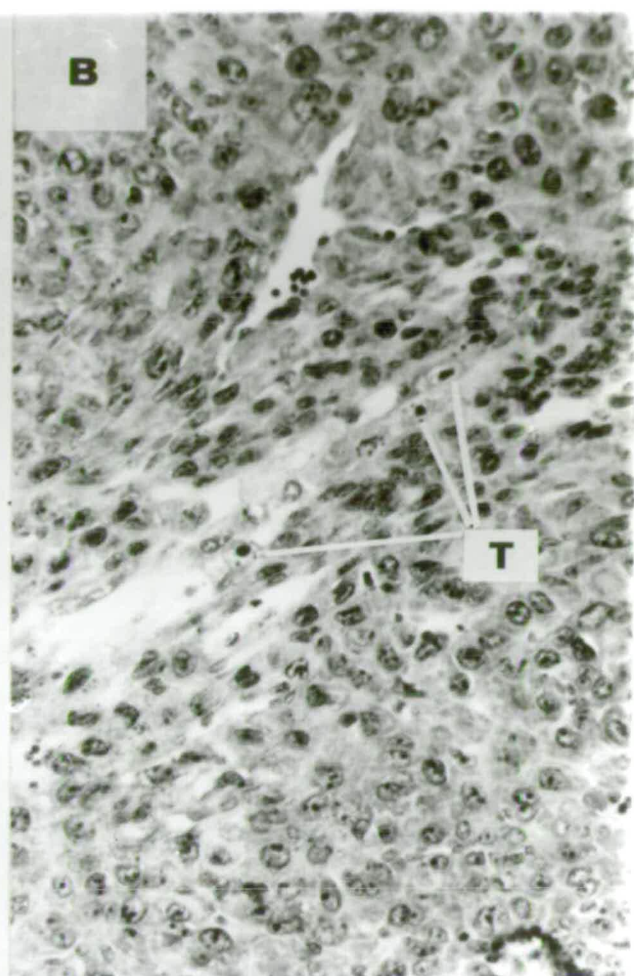
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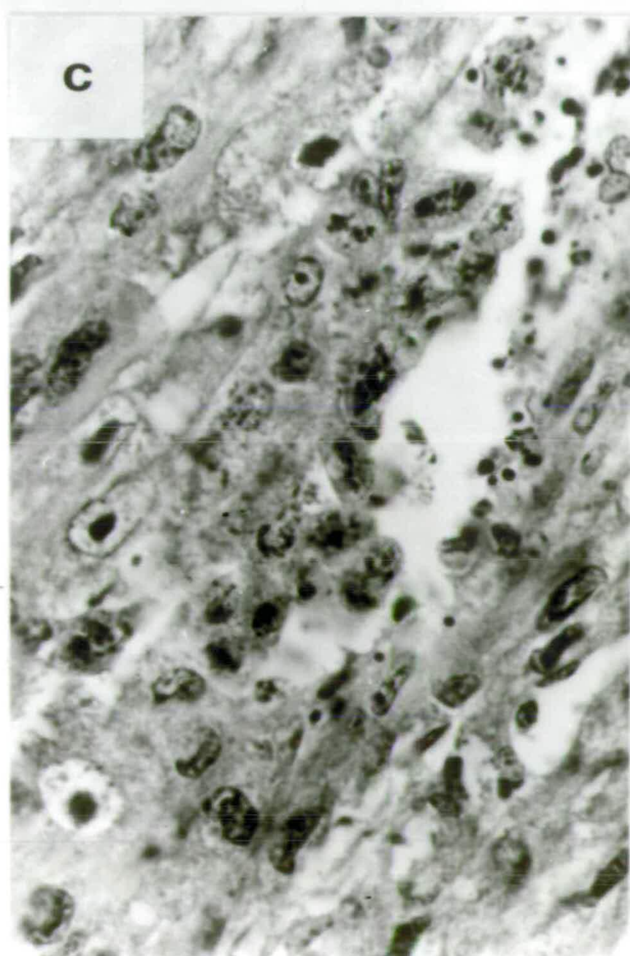

C

10 (28.6%) out of 35 embryos present in uteri from inter-heterozygote matings, however, were in a state of advanced degeneration and were starting to be resorbed. The X^2-test shows that this proportion is not significantly different from 25% (X^2 = 0.24). In all cases apparently normal local decidual responses had been induced, although the embryos themselves had become little more than disorganised masses of necrescent cells and cellular debris, barely distinguishable from the decaying remains of the detached uterine epithelium. Of those cells still intact, almost all contained chromatin fragments scattered throughout the cytoplasm in a fashion similar to that observed in the abnormal embryos of 5 days post coitum.

Of particular interest, however, was the presence of trophoblastic giant cells embedded in the decidual tissue immediately around the embryonic remains. These giant cells were a feature of all the degenerate implantation sites, and the majority appeared normal in respect of both size and morphology when compared with those of littermates.

The highly disorganised state of the great majority of cells in these embryos precluded any meaningful analysis of cell number or mitotic index, however their comparatively advanced state of degeneration makes it seem unlikely that any significant amount of growth or successful cell division had taken place during the preceding 24 hrs.

Lactational Delay (see Table 2)

Examination of 2 uteri obtained from heterozygote matings inter se and maintained in lactational delay for approximately 2 days, revealed a total of 22 embryos present. Gross morphology of all embryos was quite normal, appearing as large, elongated blastocysts lying in close

Table 2

EMBRYOS SECTIONED IN UTERO FROM Os/+ x Os/+ MATINGS AFTER 48 HOURS DELAYED IMPLANTATION

Class of Embryo	No. of Embryos	% of Total Embryos	Mean Number Abnormal/Mitotic Cells	Mean Total Cell Number	Mitotic Index
Presumptive Os/Os	3	13.6%	13.17	68 ($\pm$ 6.5)	19.37%
Littermates	19	86.4%	0.58	91.5 ($\pm$ 3.4)	0.63%)

No. of presumptive Os/Os embryos is not significantly different from 25% of total (X^2 = 1.51)

apposition to the uterine mucosa. 3 (13.6%) of these diapause blastocysts, however, contained abnormally large numbers of "mitotic" cells, constituting from one quarter to one seventh of the total cell population (mean mitotic index = 19.37%), and resembling closely the cells seen in 5 day mutant embryos. Despite the relatively low proportion of mitotic/abnormal cells in these embryos compared with their implanting (5 day) counterparts, they stood out just as strikingly on account of the particularly low mitotic index of their littermates. Indeed, the mean mitotic index of littermates was estimated to be 0.66%, with many blastocysts containing no dividing cells whatsoever.

In addition, cell counts showed that the mean cell number of the abnormal blastocysts was significantly subnormal, being calculated as 68.0 ($\pm$ 6.5) as compared with 91.5 ($\pm$ 3.4) for littermates. (Students' t-test gives $p < 0.02$).

Worthy of note, nevertheless, was the fact that non-mitotic cells in the abnormal blastocysts were morphologically normal such that the gross appearance of the embryos was not very different from that of their littermates.

While the number of abnormal embryos observed is lower than expected, the X^2-test shows that it is not significantly different from the Mendelian expectation of 25% ($X^2 = 1.51$).

Air-dried preparations

43 air-dried preparations of $4\frac{1}{2}$ day blastocysts from Os/+ x Os/+ matings were examined. In general, these consisted of large numbers of scattered nuclei, interspersed by a few metaphase spreads.

9 of the preparations, however, were very different in respect

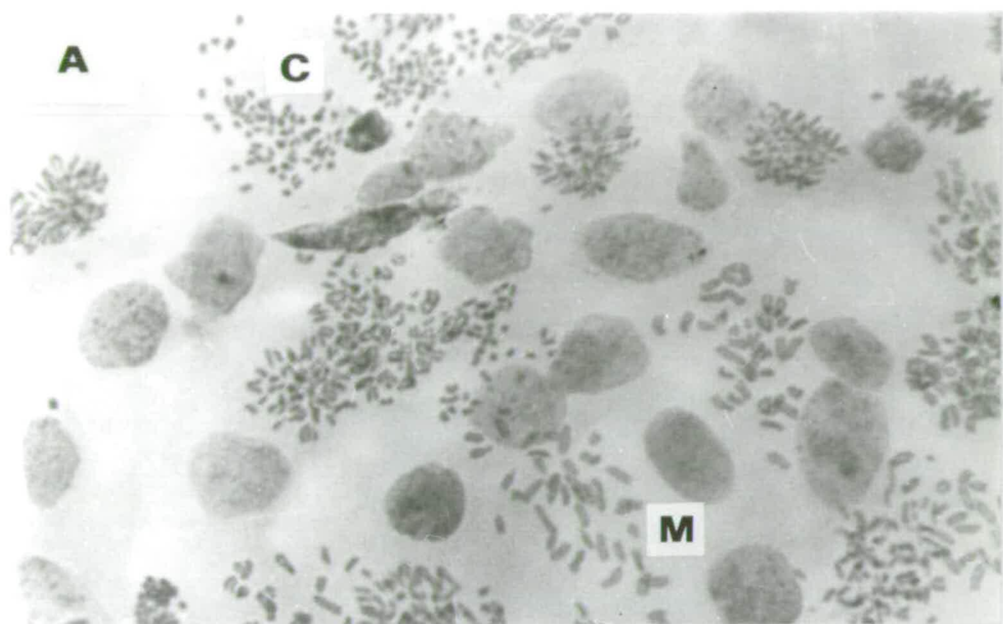
of two criteria. Firstly, they displayed an extremely high proportion of metaphase-type spreads, sometimes in such abundance that it was almost impossible to distinguish one set of chromosomes from another. Of these preparations sufficiently well spread to be countable, a mean mitotic index of 34% ($\pm$ 8.8) was calculated, in contrast to the figure of 2.26% ($\pm$ 0.4) obtained for their normal littermates. (It should be noted that mitotic indices may be prone to underestimation in these preparations, since vigorous cell spreading is apt to lead to the loss of integrity of chromosome groups). Secondly within each of these embryos, the morphology of the chromosomes differed widely, ranging through varying degrees of condensation from normal-looking metaphase spreads, to tiny blobs of chromatin scarcely larger than a centromere. Chromosomes were however of uniform appearance within any single metaphase. The more normal types of spread contained approximately 40 telocentric pairs of sister chromatids (mean = 38.6), whereas the chromatin blobs of the very highly condensed types were nearly always present in groups of about 80 (mean = 76.4). Close scrutiny of this latter kind suggested strongly that they had arisen from the separation and extreme spiralization of previously paired sister chromatids, since in some cases the chromatin blobs were still loosely associated in pairs (Plate 3). This interpretation is also supported by the fact that chromosome number never exceeded 40 in the less condensed spreads containing clearly intact chromatid pairs.

As with the sectioned material, morphology of interphase nuclei in these presumed mutant embryos appeared normal. The X^2- test reveals no significant difference between the proportion of embryos with

Plate 3 Air-dried preparations of Os/Os embryo.

A Spread showing numerous mitotic cells, with chromosome morphology ranging from normal metaphase-type (M) to highly condensed type (C). (x 500) Stained with orcein.

B Highly condensed group of chromosomes comprising 80 discrete blobs of chromatin. Signs of loose association in pairs (P) support the conclusion that previously paired chromatids have become separated during condensation. (x 3000). Stained with orcein.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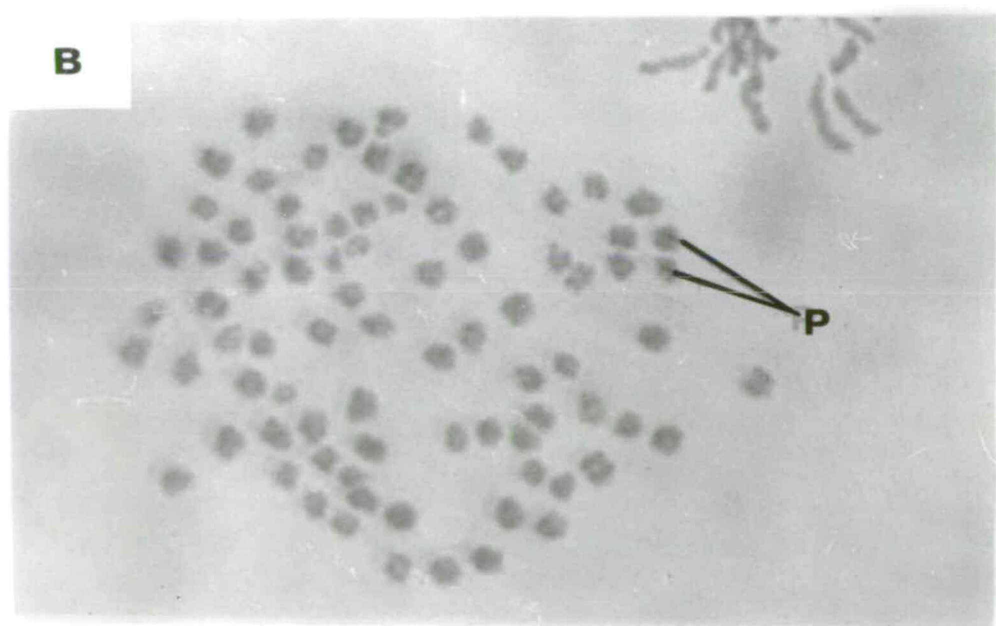

B
P

abnormally high mitotic indices and the Mendelian expectation of 25 % Os homozygotes.

Absolute cell counts could not be performed on these embryos since the degree of spreading involved in this method of preparation often caused significant loss of cells.

<u>Culture of Os/Os Embryos over the Lethal Phase</u>

(Data summarised in Tables 3 and 4)

The developmental behaviour of 149 zona-free blastocysts from Os/+ x Os/+ matings, and of 96 from Os/+ x +/+ matings was observed during culture over the lethal phase.

No abnormal class was visible in either experimental or control groups at the commencement of culture (4 days p.c.). Typically, during the next 24 hrs., embryos had become attached to the surface of the culture dish, and by 48 hrs. (6 days p.c.) were surrounded by a monolayer of outgrowing trophoblast. The inner cell mass (i.c.m) also became enlarged, and after 72 hrs. of culture (7 days p.c.) could be seen as a prominent knob protruding upwards from the centre of the trophoblast, covered by a layer of primary endoderm.

In 36 embryos (24.2%) from inter-heterozygote matings, however, this enlargement of the i.c.m. failed to occur. Indeed after 48 hrs. of culture the i.c.m. appeared markedly degenerate, and by 72 hrs. had disintegrated completely. In some cases a few necrotic cells remained clinging to the surface of the trophoblast but often only a naked layer of trophoblast giant cells remained. The morphology of these trophoblast cells seemed normal, although there was a noticeably greater uniformity of size, arising from the absence of smaller, diploid

Table 3

MORPHOLOGY OF OUTGROWTHS AFTER 72 HOURS CULTURE

Mating	Total No. of Outgrowths	No. of Outgrowths with Degenerate Inner Cell Masses	% Total with Degenerate Inner Cell Masses
Os/+ ♀♀ x Os/+ ♂♂	149	36	24.2%
Os/+ ♀♀ x +/+ ♂♂	96	2	2.1%

Proportion of outgrowths with degenerate inner cell masses in the experimental group, after correction from control data, is not significantly different from 25% (X^2 = 0.66)

or less giant, trophoblast cells which were present in the central area of littermates and controls. Viability too seemed unaffected since the cells remained healthy and were of normal appearance after a further 24 hrs. of culture (8 days p.c.)

By contrast, degeneration of the i.c.m. was observed in only 2 of the control embryos (2.1%).

After correction for the control data, the X^2 - test indicates that the number of outgrowths with degenerate i.c.m.s in the experimental group (22.1%) is not significantly different from 25% ($X^2 = 0.66$). It is thus concluded that these were Os homozygotes.

Since initial observations suggested that the trophoblastic outgrowth of these mutant embryos was somewhat smaller than normal, direct measurements were made both of the number of outgrowing trophoblast cells and of the area of outgrowth after 72 hrs. of culture. This procedure also enabled a more accurate assessment to be made of the average size of individual trophoblast cells in each group.

A total of 91 outgrowths from the experimental group (of which 21 were presumptive Os/Os), together with the 96 outgrowths from the control group, were thus analysed. The results are shown expressed as frequency histograms in Figures 1 and 2.

It can be seen that the outgrowths with degenerate i.c.m.s are considerably smaller than littermates and controls both in terms of total size and in number of trophoblast cells. Table 4 gives the mean values for each parameter in both classes of embryo, and it is apparent that the mutant class barely exceeds half the normal value in either case. Furthermore, as pointed out by Sherman & Atienza (1975), the number of trophoblast cells in normal outgrowths is likely to be marginally

Table 4

MEASUREMENTS OF OUTGROWTHS AFTER 72 HOURS OF CULTURE

Class of Embryo	Mean No. of Trophoblast Cells	Mean Area of Outgrowth	Mean Area / Trophoblast Cell *
Os/Os (21)	14.19 $\pm$ 1.37	9.14 $\pm$ 1.23	0.65 $\pm$ 0.06
Littermates (70)	27.17 $\pm$ 1.25	16.30 $\pm$ 0.68	0.59 $\pm$ 0.02
Controls ((96)	28.73 $\pm$ 0.81	17.63 $\pm$ 0.48	0.64 $\pm$ 0.02

Figures in parentheses are the number of embryos in each class.

* Mean area/trophoblast cell = $\frac{\sum \left[\frac{\text{Trophoblast cell no.}}{\text{Outgrowth area}} \right]}{n}$, where n is the number in each class.

1. Difference in mean trophoblast cell number between Os/Os and Littermates is significant at 0.1% level (t=5.4)
2. Difference in mean area of outgrowth between Os/Os and Littermates is significant at 0.1% level (t=4.9)
3. Difference in mean area/trophoblast cell between Os/Os and Littermates is not significant (t=1.24)
4. Difference in mean area/trophoblast cell between Littermates and Controls is not significant (t=1.69)

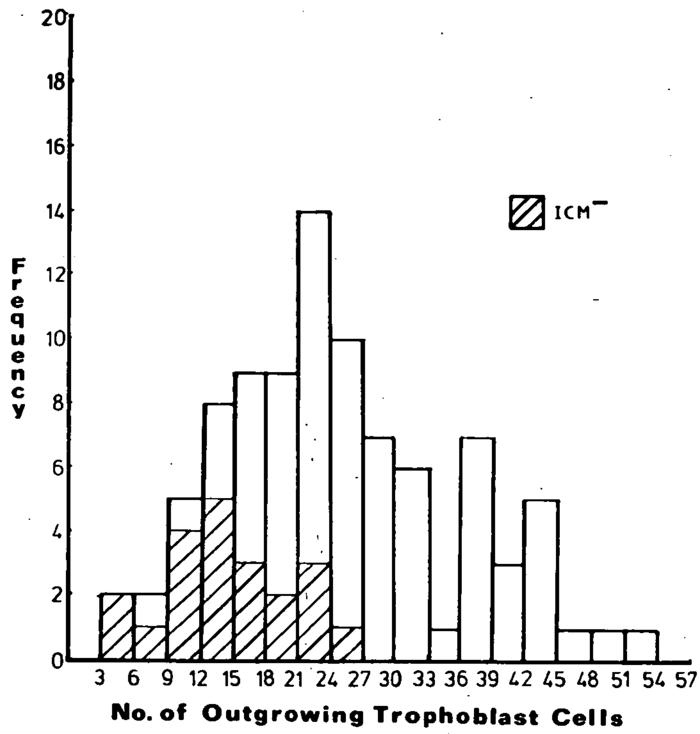
Figure 1 Distribution of trophoblast cell numbers in outgrowths after 72 hours of culture (7 days p.c.)

A Outgrowths from Os/+ x Os/+ matings

B Outgrowths from Os/+ x +/+ matings

Shaded areas show the sub-distribution of outgrowths in which the inner cell mass had degenerated.

A



B

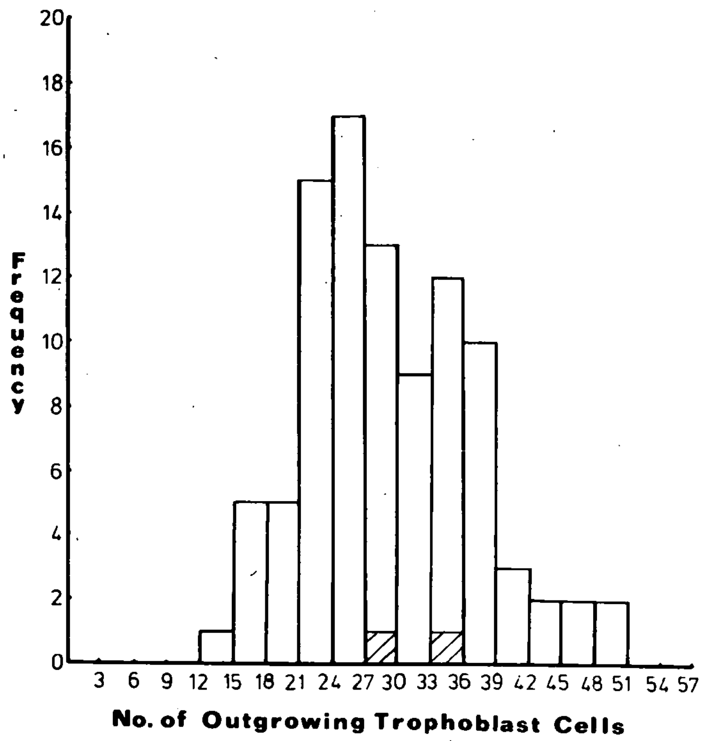


Figure 2 Distribution of the areas of outgrowth in embryos after 72 hours of culture (7 days p.c.).

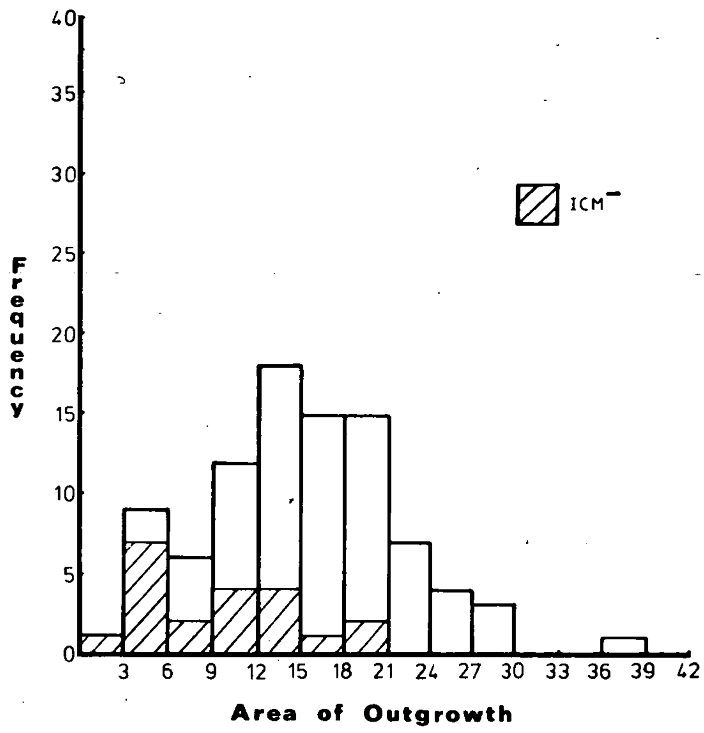
A Outgrowths from Os/+ x Os/+ matings

B Outgrowths from Os/+ x +/+ matings

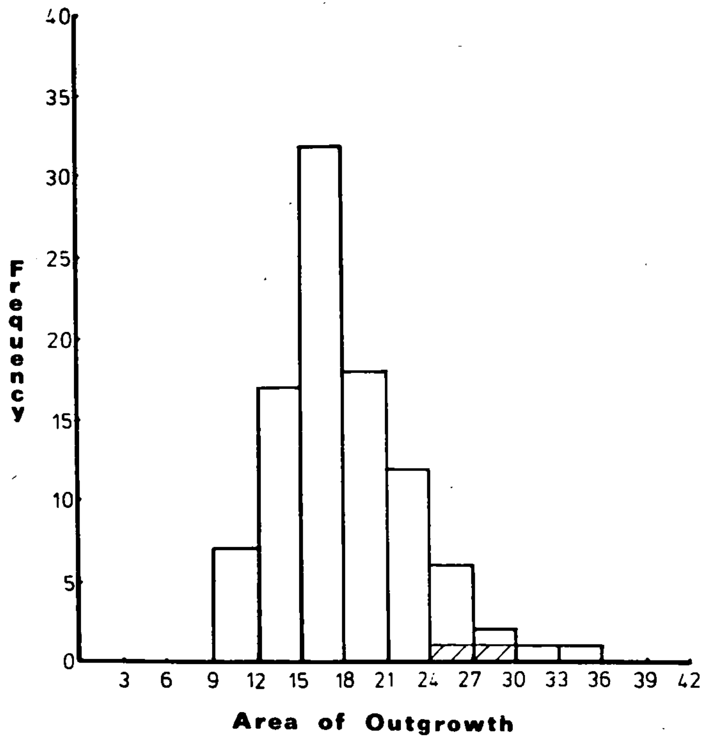
Area is represented by arbitrary units corresponding to micrometer grid squares. (1 unit = 4356 sq. microns)

Shaded areas show the sub-distribution of outgrowths in which the inner cell mass had degenerated.

A



B



underestimated using this technique due to the presence of the i.c.m. which obscures a few underlying cells.

Students' t-test shows that both mean outgrowth area and mean trophoblast number per embryo are very significantly different between mutant embryos and littermates ($p < .001$). Conversely, there is no significant difference between littermates and controls.

The histograms (Figures 1 and 2) also demonstrate that the mutant embryos form a small, discrete class within the overall distribution of the experimental group.

Despite the difference in overall size, however, there is no evidence for a reduction in mean trophoblast cell size in the mutant outgrowths as estimated from the equation.

$$\text{mean trophoblast cell size} : \quad \frac{\sum \left[\frac{\text{trophoblast cell no.}}{\text{outgrowth area}} \right]}{N}$$

(see Table 4). Indeed the trophoblast cells of the mutant embryos appear, on average, to be slightly larger than those of their littermates, although the difference does not reach formal levels of significance.

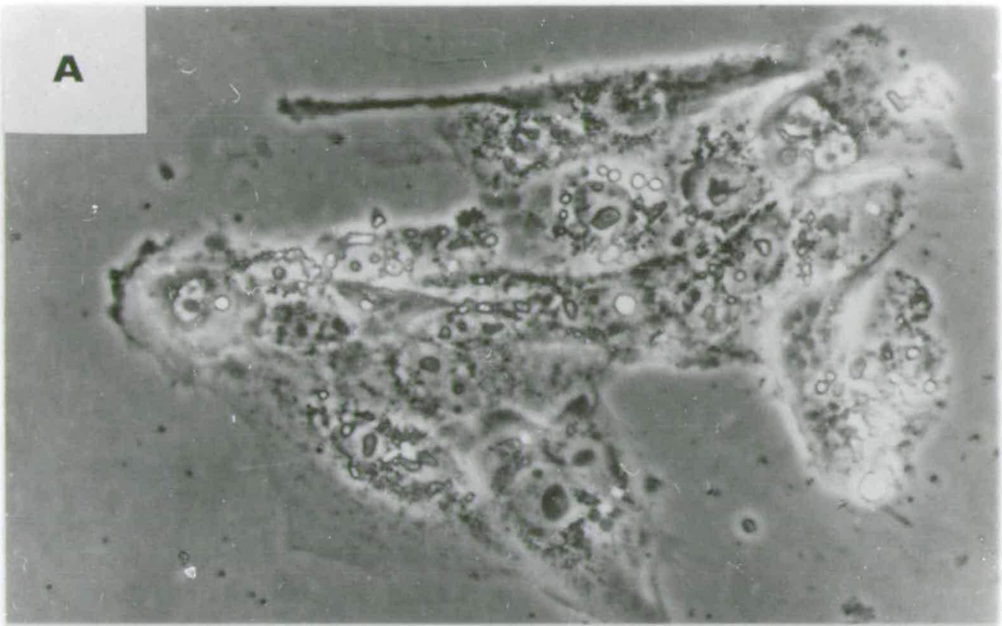
Plate 4 Embryos from Os/+ x Os/+ matings cultured over the lethal phase.

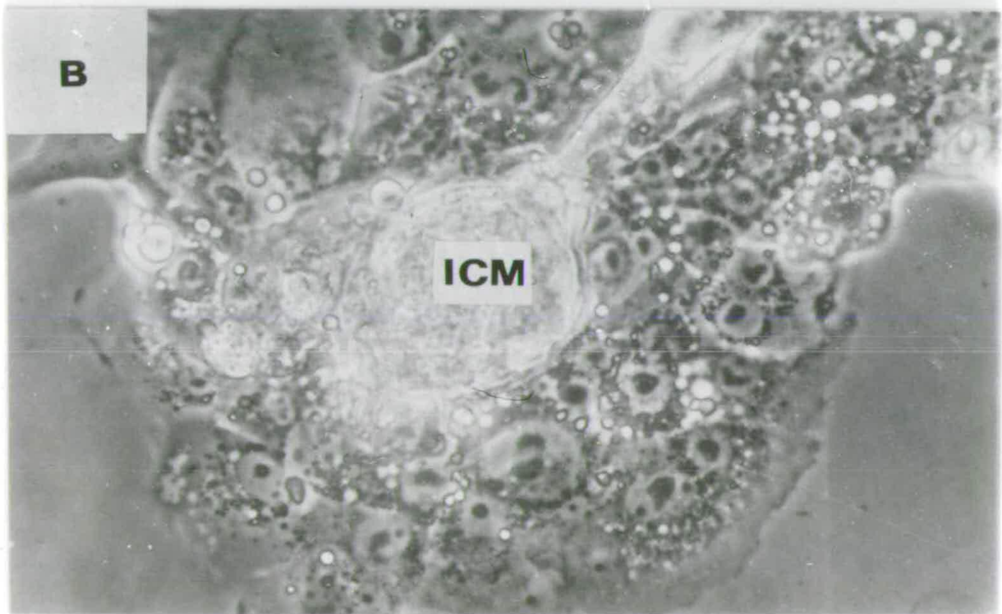
A Presumptive Os/Os embryo after 4 days of culture. The inner cell mass has disintegrated leaving only a few necrotic fragments. Trophoblastic giant cells are outgrowing normally.

B Normal littermate of A, displaying numerous small trophoblast cells close to the prominent inner cell mass (ICM). The size of the peripheral trophoblastic giant cells is comparable with those of A.

(Magnification x 300)

Phase contrast optics.


A


B
ICM

DISCUSSION

The results of the histology in this study substantially corroborate those of the original investigation performed by Van Valen (1966). In addition, however, the air-dried preparations of Os/Os embryos clearly confirm her suspicion that the abnormal cells contraining free chromatin bodies are indeed blocked in mitosis. More precisely they are unable to progress beyond metaphase. The observation that affected cells often occur in pairs, which gave rise to the alternative theory that newly formed daughter-cells may be unable to re-enter interphase, was also noted to some extent in the present study. Nevertheless, this phenomenon is equally compatible with the concept of a metaphase block. Neighbouring cells will often be sister products of a preceding division and hence will not only have shared an identical development lineage, but will also have an enhanced probability of possessing a synchronous mitotic cycle, leading to simultaneous arrest.

Cell counts of 5-day embryos rule out any possibility that the high proportion of metaphase cells seen in mutant embryos represents an increased rate of cell division, since cell number is found to be substantially reduced. The morphology of the chromosome spreads also opposes this idea; extreme chromosomal condensation as observed in Os/Os embryos is not seen in normally dividing cells but may be produced by prolonged cytostatic treatment with spindle poisons such as colchicine (Hadorn 1961). In particular it is significant that the discrepancy in mean cell number between mutant embryos and littermates (96 as opposed to 130) can almost exactly be accounted for if it is assumed that the 40% "mitotic" cells observed in the former group have been

blocked during division. These figures also support Van Valen's conclusion that the abnormality sets in during the 7th or 8th division; if the mutation affects all cells which have undergone a specific number of cycles, then allowing for the accumulation of some asynchrony during cleavage, a mean cell number of 96 indicates a blockage occurring at the 8th mitotic division.

The condition of embryos during delayed implantation also lends weight to the hypothesis that the mutation affects only dividing cells, and that its critical period is related either to the number of cell divisions undergone or to the developmental stage of the embryo, rather than to absolute time post coitum. This is demonstrated by the fact that the majority of cells in the mutant delay embryos appeared quite normal more than two days after the phenocritical phase would have occurred in a standard pregnancy. That up to 25% of cells were affected in these embryos presumably reflects the continuance of cell division which has been reported to occur during the initial phase of delay (McLaren 1968), such that cells may reach the critical stage of development and become arrested prior to entering the G1 phase in which diapause cells appear to subsequently exist. (Sherman & Barlow 1972).

Perhaps the most revealing feature of the histology, however, was seen in uteri sectioned at 6 days. Although it is not surprising that the mutant embryos should have induced a decidual response, since some initial stages of implantation have taken place on the previous day, the presence of overtly healthy trophoblastic giant cells surrounding the implantation site of an otherwise necrotic embryo is of considerable significance. Van Valen also notes briefly that "most trophectoderm

cells were normal" at this stage, and indeed two typical giant cell nuclei may be seen in one of the photographs accompanying her paper (Van Valen 1966 - Fig. F).

The behaviour of Os/Os embryos _in vitro_ almost certainly mirrors the events which take place _in utero,_ although with a small lag in absolute timing presumably resulting from slight growth retardation in culture. The persistance of giant cells after complete degeneration of the inner cell mass, in particular underlines the similarity between development _in vivo_ and _in vitro_ . There is no statistically significant difference in average size between the outgrowing trophoblast cells of mutants and littermates, although the slightly larger values obtained for mutant outgrowths may reflect the absence of the small trophoblast cells found in the central region of normal outgrowths. These may represent cells inhibited from becoming giant by their proximity to the inner cell mass,as appears likely to be the case with trophoblast cells _in vivo_ (Gardner & Johnson 1972), or may be untransformed cells of ectoplacental-cone type which have not yet become polyploid and may still undergo division. The latter interpretation would satisfactorily account for the reduced overall size and giant cell number of Os/Os outgrowths, if new trophoblast cells cannot be mitotically produced beyond the phenocritical phase, such that the surviving trophoblast is composed entirely of giant cells transformed prior to this time. It is worth noting, nevertheless, that the reduction in giant cell number seen by Ansell & Snow (1975) in outgrowths from embryos treated with [^{3}H] - thymidine, can best be attributed to early polyploidization of all trophoblast cells without significant proliferation, in the absence of an inner cell mass.

The refractoriness of the giant cells to the action of the lethal gene is most readily explained in terms of their cell cycle. Trophoblastic giant cells have been shown to contain many times the haploid DNA content (Barlow & Sherman, 1972; Zybina 1963), and analysis of enzyme variants has established that this size is not achieved by cell fusion but is a true polyploidy almost certainly resulting from endoreduplication (Chapman, Ansell & McLaren 1972; Gearhart & Mintz 1972). Furthermore, Snow & Ansell (1974) in agreement with an earlier observation by Zybina (1970) have demonstrated that after actinomycin treatment, the chromatin of giant cell nuclei will condense into discrete clumps corresponding to the diploid chromosome number, and has thus almost certainly been present in a state of polyteny. Consequently since normal mitosis does not occur in giant cells, presumably the metaphase block which traps actively dividing cells in Os/Os embryos is not encountered. Clearly trophoblast cells which have initiated endoreduplication prior to the onset of Os gene action will continue to thrive unaffected.

The exact mode of action of the gene on mitotic cells cannot be determined directly from these experiments, but for certain reasons the mitotic apparatus, and in particular the spindle, seems a likely candidate for the site of disruption. Firstly, as seen in wax sections, the chromosomes of affected cells often exhibit a much more disorganised appearance than is observed in normal metaphases, suggesting that the spindle (if present) is incapable of organising them into an orderly metaphase-plate. Secondly, outgrowths treated with colcemid, which like colchicine prevents spindle formation by inhibiting tubulin poly-

merization (Borisy & Taylor 1967; see Deysson for review, 1968), will develop into almost perfect phenocopies of cultured Os/Os embryos (Sherman & Atienza 1975). In this experiment it was found that the addition of colcemid in the culture medium to outgrowths, at a time analogous to the onset of the phenocritical phase of Os homozygotes, could result in the selective killing of the inner cell mass alone. Further, it was discovered that under certain conditions, the trophoblast was capable of withstanding concentrations of colcemid over ten times higher than were lethal for the inner cell mass. As with Os/Os embryos, outgrowths which had been treated with the drug, developed purely as inner cell mass-less monolayers of trophoblast which were morphologically normal save for the absence of smaller (diploid) cells.

Such a hypothesis does not in itself, however, readily explain the specificity of the lethal phase for the 7th or 8th cleavage. Without further biochemical investigation, any discussion of the underlying causes at the level of gene expression must be highly speculative; nonetheless, there is evidence, albeit from other sources, which makes the inability to replace or supplement compounds required for the functioning of the mitotic apparatus a feasible proposition, while remaining consistent with phase specificity. In particular, experiments performed on sea urchin eggs have demonstrated that, in this animal at least, the microtubules of which the spindle is constructed are synthesised on stored maternal messenger RNA. (Raff et al.1972; Raff & Kaumeyer 1973). This conclusion is supported by the fact that incorporation of [^{3}H]- leucine into microtubules during early cleavage is unaffected by actinomycin D (Raff et al.1971). This type of synthesis continues to

supplement a pool of tubulin dimers, which is polymerised and depolymerised with successive mitoses, until the embryos' own genome becomes capable of taking over production. If such a system does operate in the early mouse embryo, although as discussed in Section 1 the existence of stored messenger in mouse embryos has not yet been unequivocally established, it is easy to see how the effects brought about by a mutant allele such as Os may be produced. Amount of stored messenger per cell will decrease exponentially during cleavage, while requirement for its synthetic product per cell will remain constant. If the Os^+ gene codes for tubulin, or some other component or enzyme vital to the function of the mitotic apparatus which is initially synthesised on stored messenger RNA, then clearly it must take over the production of this substance before dilution/degradation of the messenger becomes critical. Consequently, in embryos with malfunctioning Os^+ genes (i.e. Os/Os) there will come a stage after a specific number of divisions, when neither stored messenger nor the embryo's own genome is capable of synthesising sufficient quantitites of the required component to form a functional mitotic apparatus, such that cleavage is arrested during mitosis. Since the amount of intact stored messenger per cell will be reduced by at least 50% with each successive cleavage, it may be expected that the phenocritical phase will be highly specific with respect to the number of cell divisions previously completed. If, as is the case with somatic mammalian cells, elements of the spindle are synthesised more than one entire cell cycle before the division in which they are actually utilized (Alov et al.1971; Kazan'ev 1971), failure of gene expression at the molecular level may occur some time before the mutant phenotype is expressed at the cellular level.

Such a hypothesis, postulating failure of the embryonic genome to take over production of a component of the mitotic apparatus from a rapidly dwindling concentration of maternal messenger, accounts satisfactorily for all the observed phenomena, associated with homozygosity for Os, of phase specificity, disordered mitotic chromosomes, and insusceptibility of endoreduplicating cells. Electron microscopy may prove able to demonstrate any structural peculiarities of the microtubules in the mutant embryos during the lethal phase, as has been successfully shown for certain groups of cells in the partially analogous lethal polyploid mutant of Drosophila hydei (Rungger-Brandle 1976). The increasingly sensitive methods now available for separating embryonic proteins and examining their synthesis may help to elucidate any possible role of stored messenger RNA in the mitotic function of both normal and Os/Os embryos.

SECTION 4

A STUDY OF EMBRYOS HOMOZYGOUS FOR TAIL-SHORT (Ts)

INTRODUCTION

The mutation tail-short (Ts) arose spontaneously in laboratory stocks in 1946 (Morgan 1950). Mice heterozygous for Ts are clearly distinguishable by their small size and short kinked tails, although both Morgan and Deol (1961) have described numerous other skeletal defects associated with this allele, including shortening of the forelimb and triphalangy. Expressivity is very variable even between mice of the same inbred strain (Deol), but the effects of the mutation have been shown by Morgan to be susceptible to dramatic modification when introduced into different genetic backgrounds, ranging from complete absence of tail with reduced viability, to total lethality. Deol has traced the skeletal abnormalities of Ts/+ mice back to the 11th day of gestation at which time distortions of the neural-tube and notochord are visible. In addition, a marked anaemia accompanies development, which can be identified as early as the 8th day by a reduction in the number of blood-islands in the yolk sac, but which ultimately disappears during the last few days of pregnancy. It is not clear to what extent the skeletal and haematological defects are interconnected, but Deol has suggested that the anaemia may lead to differential retardation of growth in simultaneously developing structures, presumably linked to their degree of dependence on vascularisation. According to this hypothesis, deformation of the tail may arise as a result of the notochord becoming kinked through elongating more rapidly than the neural tube.

Ts has not yet been genetically mapped. Its effect on tail length caused Morgan (1950) to speculate that the mutation might be in some way related to the T-locus, and to remark on the similarity between the

altered severity of expression of Ts when present in different inbred backgrounds, and the interaction of T with the recessive t-alleles. No formal account, however, of genetic studies designed to test this possibility have been published.

The breeding data obtained in the original study by Morgan, led him to conclude that Ts was a prenatal lethal in the homozygous condition. Deol subsequently reported that the average number of implantations found in Ts/+ females pregnant by Ts/+males was only three-quarters of that found in Ts/+ females mated to +/+ males, indicating that the mutant homozygotes die before or shortly after implantation. No detailed analysis concerning the recessive lethality of Ts has until now been performed, and it has been the purpose of this study to determine the exact stage and manner of death of the homozygotes. An histological analysis in utero of Ts/Ts embryos, spanning the implantation period, is presented together with observations of their behaviour in vitro when cultured over the lethal phase.

MATERIALS AND METHODS

Experimental Animals

The mice used in this study were of the BALB/c strain maintained in the Department of Genetics, University College London. These stocks were kindly made available by Dr. M. S. Deol.

Histological Analysis

The following matings were employed:

Ts/+ ♀♀ x Ts/+ ♂♂ - experimental group to provide Ts/Ts embryos

Ts/+ ♀♀ x +/+ ♂♂ - control group to facilitate identification of non-specific abnormalities.

The procedure of using heterozygote females (rather than males) in the control matings was adopted to ensure that any unknown effects of the mutation on the maternal reproductive system, which might complicate the analysis, would be equalised in both groups.

Pregnant females aged 8-14 weeks were killed by cervical dislocation at approximately 64, 88, 112 or 136 hours *post coitum* as determined by the vaginal plug method. Oviducts (64 hrs. only) or uteri were excised, fixed in Carnoy's or Bouin's fluid and embedded in paraffin wax. Serial sections were cut at 6µm, 7½µm or 10µm, and stained with haematoxylin and eosin.

Cell numbers were determined from serial sections as described in Appendix 1, according to the section thickness of each preparation.

Autoradiography

Uptake and incorporation of [^{3}H] - uridine were examined in embryos from Ts/+ x Ts/+ matings in order to determine if RNA

synthesis was taking place during the stage leading up to their final arrest.

Embryos were flushed from the uterus at approximately 88 hours post-coitum with warm PB1 medium (Whittingham & Wales 1969) washed twice and incubated for 1 hr. in routine ova culture medium (Whittingham 1971), containing 2.5 μCi/ml. /5-^{3}H_7- uridine (Specific activity 5 Ci/mmol Radiochemical Centre, Amersham). After three 5 minute washes in PB1, they were further exposed to a 5 hour "chase" in medium containing 5 mgms/ml unlabelled uridine (Sigma). The embryos were then fixed in Bouin's fluid, encapsulated in wax and sectioned at 6μm. Serial sections were mounted on subbed slides, covered with Kodak AR10 autoradiographic stripping film and exposed at 4°C for 14 days. After development, the autoradiographs were photographed and the stripping film removed gently in warm water. Sections were then stained with Ehrlich's haematoxylin and aqueous eosin, so that cytological detail in the specimens could be compared with the autoradiographic grain pattern.

Culture over the Lethal Phase

The following matings were employed:

Ts/+ ♀♀ x Ts/+ ♂♂ - experimental group

+/+ ♀♀ x Ts/+ ♂♂ - control group

The use of wild-type females in the control matings was adopted as a means of maximising the animal stocks available, since the results of the histological analysis had shown no perceptible maternal effect of the Ts allele on the early development of viable embryos.

Pregnant mice were killed by cervical dislocation at 83-84 hrs post coitum. Uteri were dissected out and the contents flushed into a

watchglass with warm PB1 medium. Embryos thus obtained were washed, scored as either morulae or blastocysts and transferred to pre-equilibrated droplets of Eagle Minimal Essential Medium (Flow Labs) supplemented with 20% foetal calf serum, 1.6 mM glutamine and 100 units/ml penicillin. Culture droplets were suspended in paraffin oil in Falcon plastic dishes, and maintained at 36.5 ^{o}C in an atmosphere of 5% CO_2 in air.

Embryos were examined after 24 and 48 hrs of culture, under bright field and phase contrast illumination on a Wild M40'inverted' microscope, and scored for developmental prowess according to the following categorisation:

Morula : no sign of cavitation visible.

Blastocyst : blastocoele or initial signs of cavitation visible.

Undeveloped: no significant change in developmental stage or morphology from that observed prior to culture.

Expanded : blastocyst with enlarged blastocoele and tightly stretched, translucent trophectoderm.

Hatched : blastocyst which has freed itself substantially or totally from the zona pellucida.

Outgrowth : embryo which has become firmly attached to the surface of the culture dish, and in which the trophoblast cells are migrating outwards over the plastic surface.

Karyotype Analysis

Chromosome spreads were prepared according to the method of Evans et al.(1972), from the tail-tips of 13 day Ts/+ embryos, which

can be clearly identified at this stage by tail length (Deol 1961). Chromosome spreads thus obtained were G-banded by the method of Seabright (1971), by a 15-45 second immersion in 0.25% trypsin dissolved in PBS, followed by 10 minutes in Geimsa stain (Gurr) diluted in 1:20 in Sorensen's buffer (pH 6.9).

Ideograms were constructed from high-power photomicrographs of well banded spreads.

RESULTS

Histological Analysis

(Data summarised in Table 1)

$2\frac{1}{2}$ days p.c.

6 oviducts from Ts/+ x Ts/+ matings, containing a total of 52 embryos, were examined at this stage. 3 single cell ova, presumed to be unfertilised, and 2 normal-looking 4-cell embryos were present in this group, but no morphological abnormalities could be seen amongst the remainder. Excluding the unfertilised ova, the mean cell number was estimated as 7.26 ($\pm$ 0.22).

No evidence of a 3:1 dichotomy with respect to cell number was detected amongst this group, and it is thus concluded that Ts/Ts embryos are not distinguishable, in terms of gross morphology, from their littermates at this stage.

$3\frac{1}{2}$ days p.c.

8 uteri from Ts/+ x Ts/+ matings and 9 uteri from control matings were studied, containing 61 and 75 ~~abnormal~~ embryos respectively.

A distinct class of 17 (27.87%) abnormal embryos was apparent in the experimental group. These were distinguishable from their littermates by several criteria:

(a) Absence of cavitation : no sign of blastocoele formation could be seen in this class, in contrast to most of their littermates which exhibited a well developed cavity.

EMBRYOS SECTIONED IN UTERO FROM Ts/+ x Ts/+ and Ts/+ x +/+ MATINGS

Mating	Days p.c.	No. Uteri	Total No. Embryos	No. Abnormal	No. Presumptive Ts/Ts	Mean Total Embryos/ Litter	Mean Normal Embryos/Litter
Ts/+ ♀♀ x Ts/+ ♂♂	$2\frac{1}{2}$	6	52	3	-	8.66 ± 1.20	8.16 ± 1.19
	$3\frac{1}{2}$	8	61	17	17	7.63 ± 0.26	5.50 ± 0.59
	$4\frac{1}{2}$	10	77	12	12	7.70 ± 0.37	6.50 ± 0.50
	$5\frac{1}{2}$	10	67 (69 dec.)	7	4	6.70 ± 0.54	6.00 ± 0.63
Ts/+ ♀♀ x +/+ ♂♂	$2\frac{1}{2}$	-	-	-	-	-	-
	$3\frac{1}{2}$	9	75	2	-	8.33 ± 0.47	8.11 ± 0.42
	$4\frac{1}{2}$	8	67	1	-	8.37 ± 0.50	8.25 ± 0.45
	$5\frac{1}{2}$	7	60 (60 dec.)	0	-	8.57 ± 1.08	8.57 ± 1.08

Figures in parentheses are the total number of decidual swellings

See text for tests of "significance"

(b) Pale staining : the cytoplasmic staining of these embryos was a striking feature of their appearance, seen as a pale eosinophilic pink, resembling that of 2-8 cell embryos. Littermates and controls, whether late morulae or blastocysts, were much darker in appearance, presumably reflecting a greater affinity for haematoxylin.

(c) Low cell number : this characteristic was sufficiently marked to be evident at first sight, the abnormal class having an estimated mean cell number of 13.74 ($\pm$ 0.48) as opposed to 30.29 ($\pm$ 1.05) for littermates.

(d) Nuclear morphology: the nucleoli of these embryos often appeared very ragged in outline and irregularly distributed throughout the nucleoplasm when compared with the more dense, compacted morphology of those of their littermates. This was not sufficiently consistent, however, to be reliably diagnostic of the class.

All abnormal embryos exhibited features 'a', 'b' and 'c', and in over half, 'd' was also noticeable.

Only 2 (2.67%) of the 75 embryos in the control group were

distinctly abnormal - one unfertilised, the other consisting of a densely staining mass of crowded necrotic cells. 4 (5.33%) control embryos had not yet cavitated, but showed normal staining capacity, cell number and nucleolar morphology.

The X^2-test indicates that the proportion of abnormal embryos in the experimental group is very significantly higher than in the control group. ($X^2 = 16.2$, $p < 0.001$). In addition, the number of abnormal embryos in the experimental group (27.87%) conforms well with the expected Mendelian ratio for mutant homozygotes of 25% ($X^2 = 0.27$ - not significant).

It is therefore assumed that the abnormal embryos are tail-short homozygotes.

$4\frac{1}{2}$ days p.c.

Of the 77 embryos examined in 10 uteri from Ts/+ x Ts/+ matings, 12 (15.58%) exhibited abnormalities consistent with having developed from the mutant class seen at $3\frac{1}{2}$ days post coitum. Once again these were distinctively pale staining and very strikingly retarded both in developmental stage, and in cell number (mean cell number $= 21.85 \pm 1.61$, for intact embryos). Irregularly shaped nucleoli were also still a common feature, but 4 out of the 12 had succeeded in cavitating, to form small primitive blastocysts. There was no clear delineation into inner cell mass and trophoblast in these embryos, probably due to the small number of cells present, almost all of which retained contact with the exterior surface. The remaining 8 were still morulae of which 3 appeared incomplete and presumably had partially disintegrated. The zona pellucida seemed still to be present around at least 2 of the intact abnormals as judged by their

particularly rounded appearance, and by the even circumferential gap between themselves and the surrounding epithelium. In some cases the embryos were totally encased within crypts, snugly surrounded by intact cuboidal epithelium; in other cases there appeared to be a much less intimate association, with the embryos lying more or less free in the narrowing uterine lumen. No signs of invasiveness were observed in any of these embryos, nor was there any evidence of the initiation of a local decidual reaction.

Littermates, by comparison, were expanded blastocysts with a mean cell number of 97.86 ($\pm$ 3.92). Most were undergoing attachment to the uterus via the polar trophoblast, and in many cases an incipient local decidual response had been induced.

Only 1 (1.49%) of the 67 control embryos examined was morphologically abnormal - a small, retarded blastocysts showing no signs of uterine attachment.

It will be seen that the proportion of abnormal embryos in the experimental group (15.58%) is somewhat lower than the expected 25% although the discrepancy is not formally significant ($X^2 = 3.64$). It is also evident, however, that the number of normal embryos per litter is significantly smaller than in the control groups, averaging only 6.50 ($\pm$ 0.50) as opposed to 8.25 ($\pm$ 0.45) ($t_{16} = 2.5$, $p < 0.025$), and thus close to the expected 75%. It therefore seems likely that some Ts/Ts embryos may have disintegrated completely during the previous 24 hours, or have become so degenerate as to be quite unrecognisable.

<u>5½ days p.c.</u>

By this time normal embryos had reached the egg cylinder stage

and had become attached to the uterus over most of the trophoblast surface. Invasive giant cells were numerous, and a marked decidual swelling was present around each implantation site.

Of the 67 embryos in 10 uteri from Ts/+ x Ts/+ matings, 7 (10.45%) were clearly abnormal, although 3 of these were far too advanced in developmental stage and cell number to be compatible with having developed from Ts/Ts embryos of the type seen at $4\frac{1}{2}$ days *post coitum*, and as such almost certainly were the consequence of non-specific developmental disturbances. Of the remaining 4, all of which were surrounded by comparatively feeble decidual swellings, 2 consisted of nothing more than disintegrating clusters of cells presumed to be of embryonic origin, and 2 were small, degenerate, partially collapsed blastocysts of approximately 30 cells each. The abnormalities of the latter two embryos were only partially consistent with those of the mutant homozygotes of earlier stages, one exhibiting the typically pale cytoplasm but conversely containing particularly smooth, rounded nucleoli, the other having normal nucleoli and rather heavily stained cytoplasm. In both cases the uterine epithelium, which was still present, had been partially eroded by a few primary invasive cells. In addition to the abnormal embryos, 2 small decidual swellings were observed in which no sign of embryonic tissue could be found.

7 uteri from control matings contained 60 embryos in which no major abnormalities could be detected. No empty decidual swellings were present.

The mean number of normal embryos per uterus in the experimental matings was 6.00 ($\pm$ 0.63) compared with 8.57 ($\pm$ 1.08) for

Plates 1 and 2

Embryos from Ts/+ x Ts/+ matings sectioned _in utero_ on consecutive days of development.

A $3\frac{1}{2}$ day Ts/Ts morula. Note the pale cytoplasm and irregular nucleoli (I). One nucleus is pycnotic (P) although this is not typical.

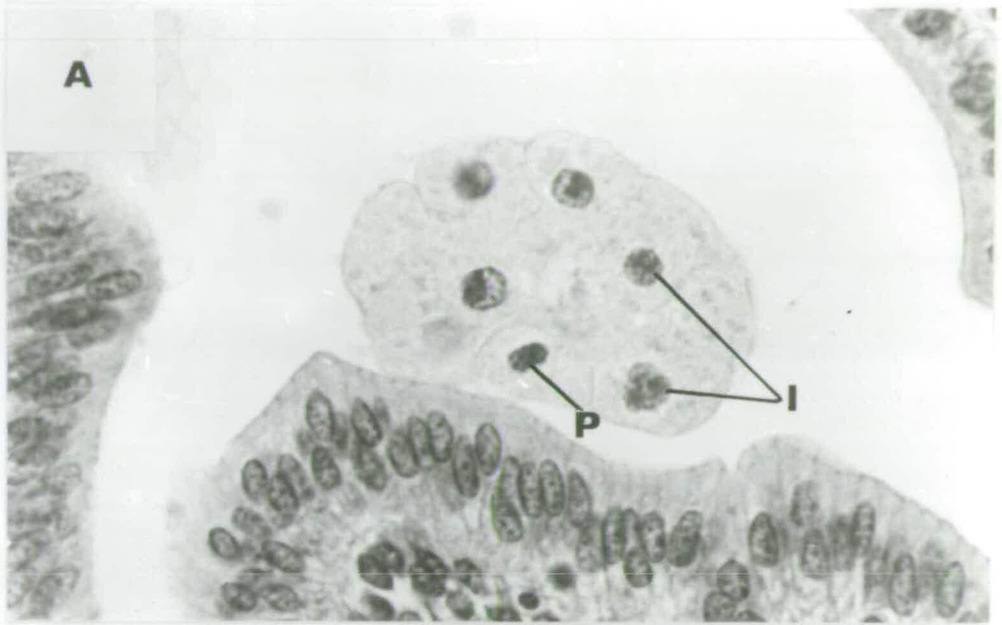
B Normal $3\frac{1}{2}$ day embryos, with well defined blastocoele, higher cell number and more darkly staining cytoplasm .

C $4\frac{1}{2}$ day Ts/Ts embryo. A cavity (C) is present but cytoplasm is still very pale and cell number low. The even space around the circumference of the embryo (Z) may mark the continued presence of the zona pellucida.

D&E Abnormal embryos of indeterminate genotype present in uteri from Ts/+ x Ts/+ matings at $5\frac{1}{2}$ days. Note the rounded nucleoli in D and primary invasive cell with W-body (PI).

(Magnification x 500)

All sections stained with H & E.


A
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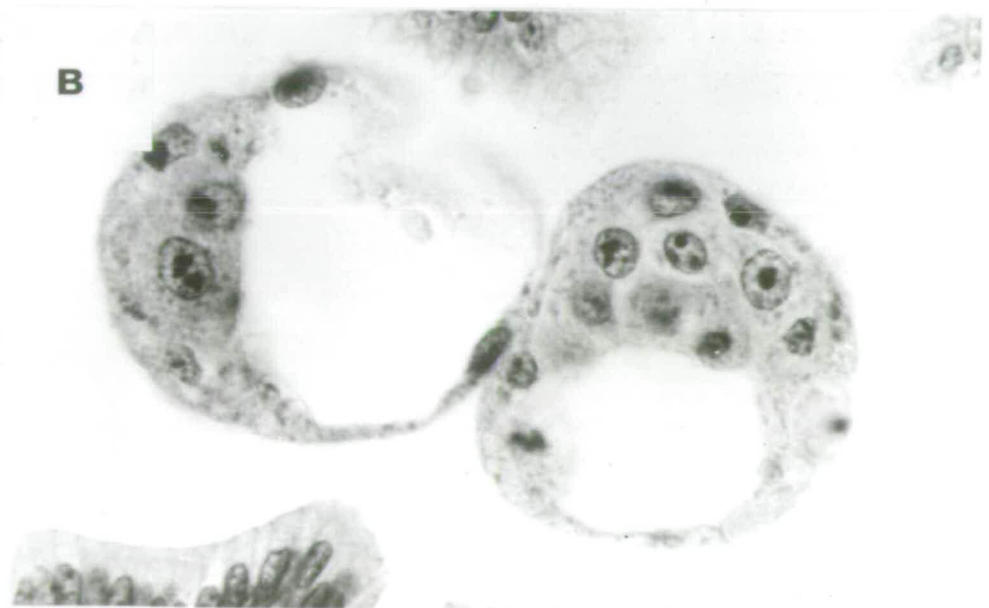
I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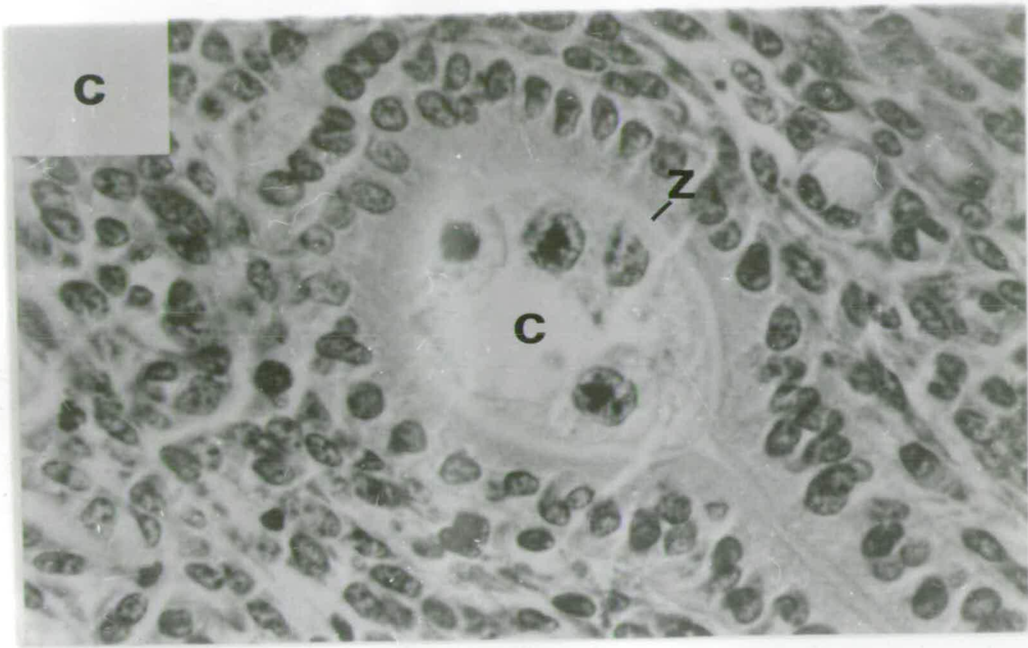
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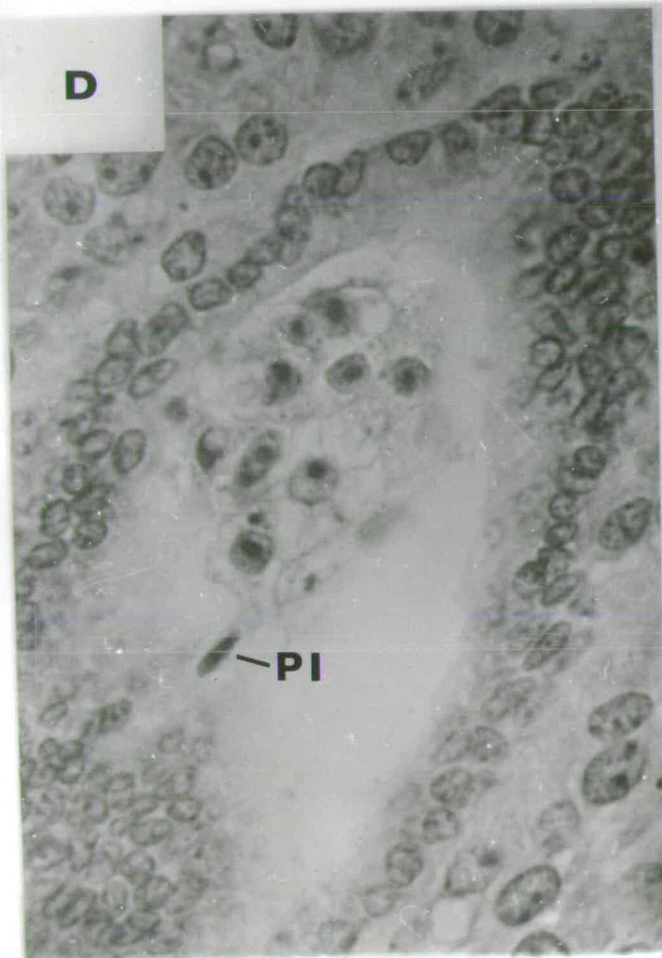
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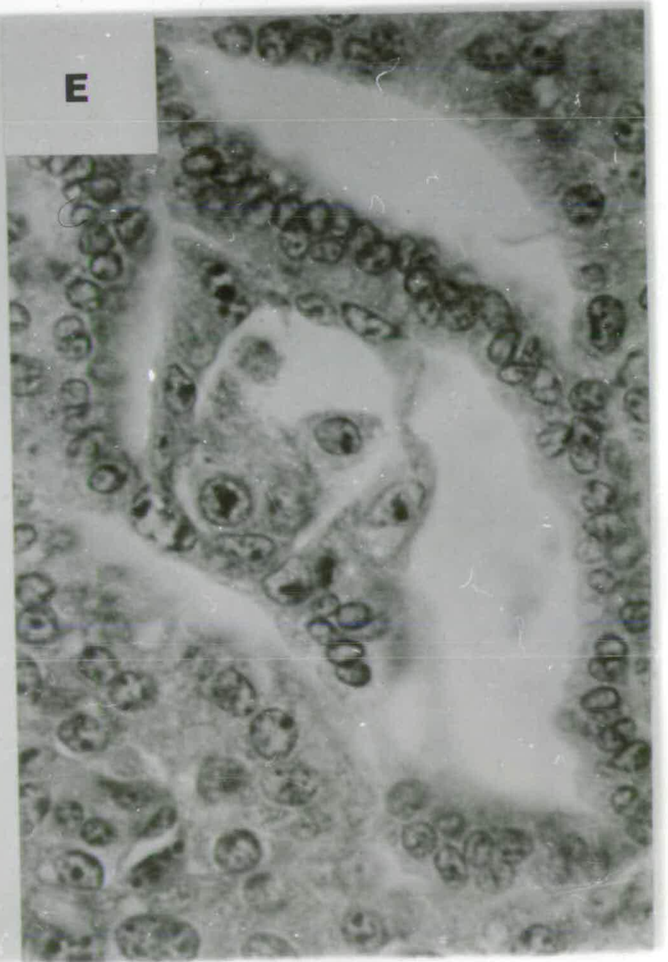

E

controls. This represents a ratio of 70%, in reasonable agreement with the Mendelian expectation of 75%.

Cell Numbers

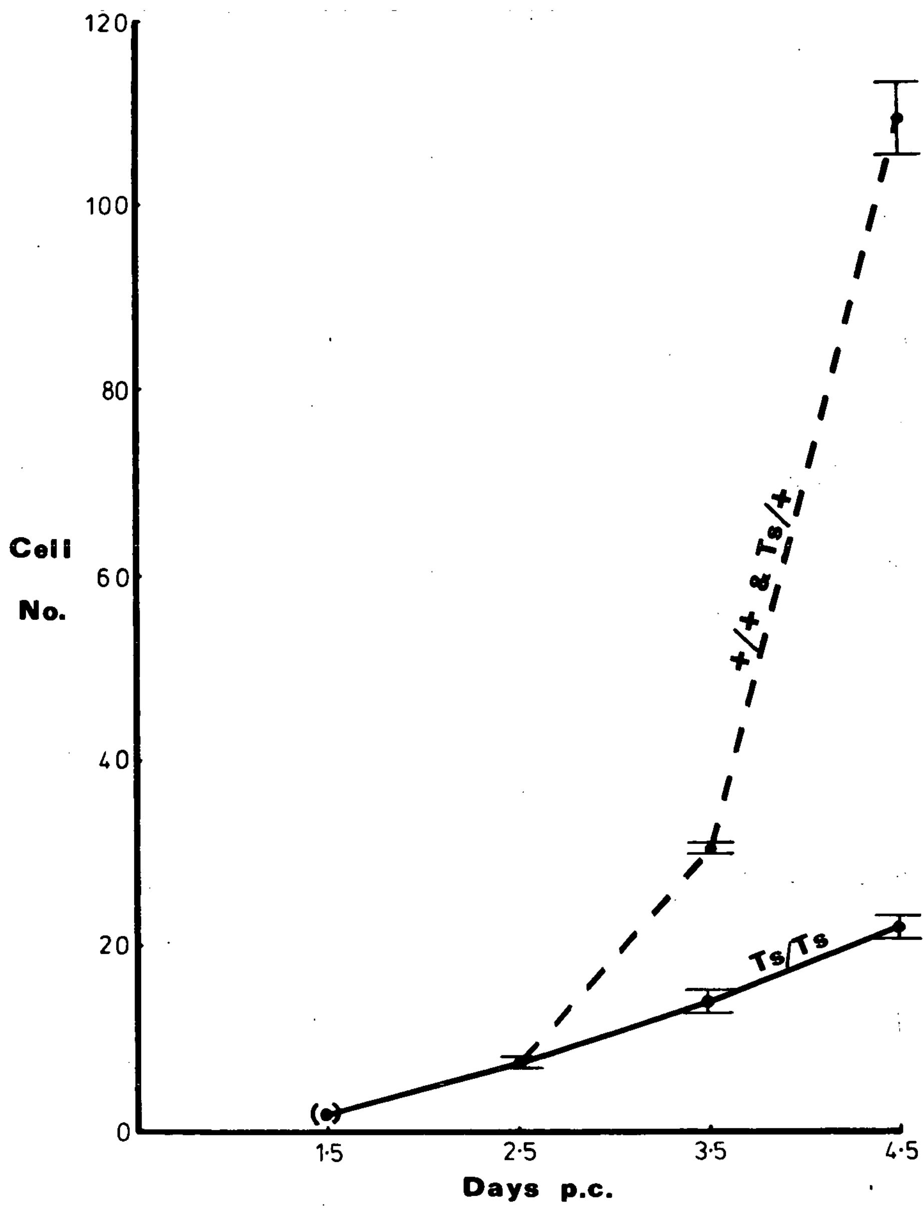
Mean cell numbers for Ts/Ts embryos and littermates are plotted in Fig. 1. It can be seen that the mutants are still capable of some cell division after they have become morphologically identifiable, but at a much reduced rate such that by $4\frac{1}{2}$ days they have not yet attained the cell number of normal $3\frac{1}{2}$ day embryos. Data from $5\frac{1}{2}$ days have not been included in the graph since it is not clear whether the abnormals observed at this stage were Ts homozygotes or merely non-specific stragglers. It is possible however that in a few cases ($<10\%$) some cell division may continue until $5\frac{1}{2}$ days.

Autoradiography

Out of 15 embryos incubated in [^{3}H]- uridine, aged approximately 94 hrs. at the time of fixation, 5 were found to have a very strikingly reduced grain count when compared with littermates (plate 3). Grain density over the latter was often so high as to preclude accurate comparative counts, but generally grain number appeared to be at least 5 times higher than in the former group. Subsequent staining, after removal of the stripping film showed that all 5 of the embryos with low uridine uptake and incorporation were typical Ts/Ts morulae displaying low cell number, pale cytoplasm and some ragged nucleoli. Nevertheless, RNA synthesis appeared to be taking place in both normal and mutant embryos as judged by the relatively high grain density over the nucleoli, and lower, more even distribution over the cytoplasm.

Figure 1 Mean cell numbers of Ts/Ts embryos and of littermates on sequential days of development *in utero*, as estimated from serial sections.

(Horizontal bars represent standard errors).



Cell
No.
120
100
80
60
40
20
0
1·5
2·5
3·5
4·5
Days p.c.
+/+ & Ts/+
Ts/Ts

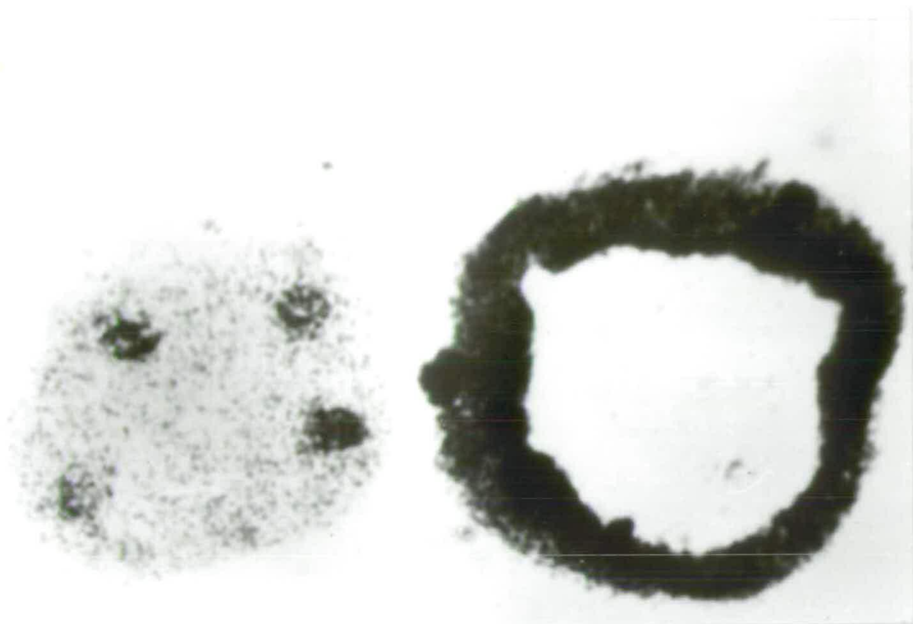
Plate 3 Uptake and incorporation of [^{3}H] - uridine in $3\frac{1}{2}$ day embryos from Ts/+ x Ts/+ matings.

A Autoradiograph of Ts/Ts morula (left) and littermate blastocyst. Nucleoli and cytoplasm are both clearly labelled in the Ts/Ts embryo although at a much lower level than in the littermate.

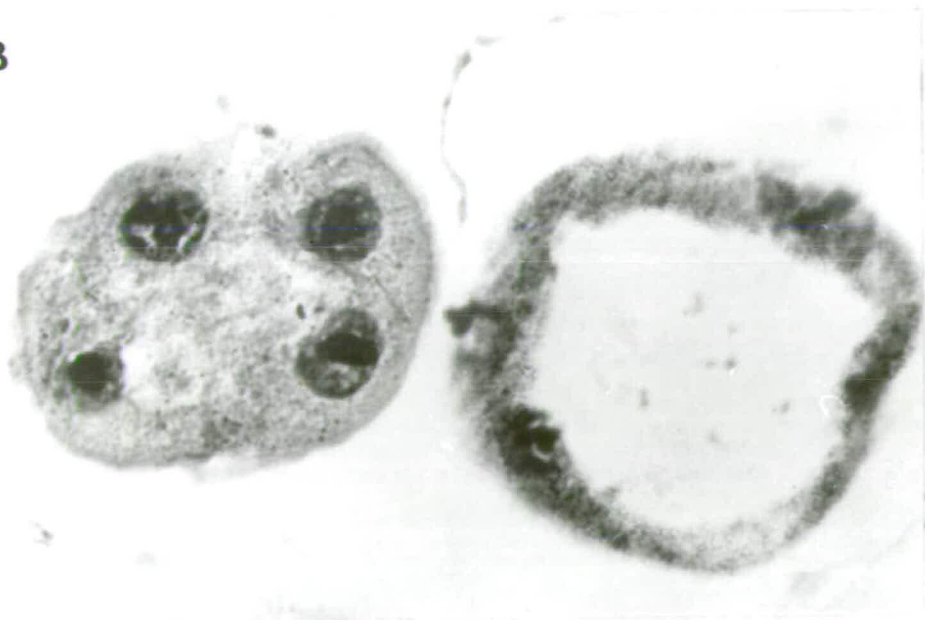
B Same embryos as in A, stained after removal of the autoradiographic stripping film. The morphology of the embryo on the left confirms Ts/Ts genotype.

(Magnification x 750)

A



B



Both normal and irregular nucleoli in the mutant homozygotes were observed to be labelled, and there did not seem to be evidence of reduced synthesis or exportation of RNA associated with the abnormal type.

In the absence of carefully timed control experiments, it is impossible to determine if RNA synthesis in Ts/Ts embryos as a whole is lower than would be expected for normal morulae of a comparable developmental stage. Nevertheless, allowing for their state of development, it is possible that the mutants are not very different from normal with respect to RNA turnover.

Culture over the Lethal Phase

(Results are summarised in Table 2)

Start of culture (3½ days p.c.)

72 embryos from Ts/+ x Ts/+ matings and 70 embryos from control matings were flushed from their uteri at approximately 84 hours *post coitum*. The majority had already cavitated to form early blastocysts; however, 32 (44.44%) of the experimental group were still morulae, compared with only 13 (18.57%) of the control group. Save for this discrepancy , it was not possible to detect any discrete, abnormal class at this stage.

The X^2-test shows that the difference in the proportion of morulae present between experimental and control groups is highly significant ($X^2 = 5.76$, $p < 0.025$).

24 hrs culture (4½ days p.c.)

After one day of culture most embryos had become fully formed

expanded blastocysts, with some even showing initial signs of hatching from the zona pellucida. 19 (26.4%) of the experimental group, however, were classified as "undeveloped" since they had failed to expand and still resembled $3\frac{1}{2}$ day embryos on morphological criteria. 12 of these showed some small degree of cavitation, but all had been categorised as morulae at the start of culture. Such cavitated "undeveloped" embryos were rarely entirely normal in appearance compared with healthy blastocysts of similar developmental stage. There was often no clear distinction between trophoblast and inner cell mass, with cells remaining rounded and relatively undifferentiated; surface vesiculation was also commonly present. Despite their retardation, only one embryo showed visible signs of necrosis.

By contrast only 2 (2.9%) of the control group had not developed normally. The experimental group thus displayed a 23.5% greater proportion of developmental failure than did the control group, after the first day of culture.

<u>48 hrs culture ($5\frac{1}{2}$ days p.c.)</u>

After 48 hrs of culture, 46 of the 53 blastocysts in the experimental group which were "expanded" on the previous day, had hatched wholly or partially from the zona pellucida, and many had become attached to the surface of the culture dish. 7 of the 'expanded' blastocysts had not yet succeeded in penetrating the zona. All 19 embryos classified as "undeveloped " on the previous day had remained "undeveloped" , although one more had formed a small blastocoele. 7 of these were now in a state of partial necrosis, displaying ballooning vesicles around their surface in a manner characteristic of dying embryos.

Table 2

CULTURE OF $3\frac{1}{2}$-DAY EMBRYOS FOR 72 HOURS

Mating	0 HOURS Blastocysts	0 HOURS Morulae	24 HOURS Expanded	24 HOURS Undeveloped	48 HOURS Hatched	48 HOURS Expanded	48 HOURS Undeveloped	72 HOURS Outgrowth	72 HOURS Expanded (Collap-sed)	72 HOURS Undevelo-ped (Deac
Ts/+ x Ts/+	40	32	53	19	46	7	19 (13 cav.)	47	6	19
(n = 72)	55.6%	44.4%	73.6%	26.4%	63.9%	9.7%	26.4%	65.3%	8.3%	26.4%
+/+ x Ts/+	57	13	68	2	64	4	2	64	4	2
(n = 70)	81.4%	18.6%	97.1%	2.9%	91.4%	5.7%	2.9%	91.4%	5.7%	2.9%

1. Difference between proportions of embryos outgrowing in experimental and control groups is highly significant ($X^2 = 14.22$, $p < 0.001$).

2. Proportion of embryos failing to outgrow in experimental group, after correction from control data, is not significantly different from 25% ($X^2 = 0.05$).

72 hrs culture ($5\frac{1}{2}$ days p.c.)

By this time, all of the embryos in the experimental group which had successfully escaped from the zona pellucida were outgrowing vigorously (65.28%), including one of those which was unhatched on the previous day. The remaining 6 of the previously "expanded" but unhatched blastocysts had now collapsed within their zonas. All 19 of the "undeveloped" group were now dead or degenerating.

In the control group, 64 (91.42%) embryos were outgrowing. None of the unhatched blastocysts had succeeded in penetrating the zona, and were now collapsed. Both "undeveloped" embryos had started to disintegrate within their zonas.

It is thus evident that amongst the embryos from Ts/+ x Ts/+ matings there was a net total, above control levels, of 26.14% failure to hatch and outgrow, of which the majority had remained relatively undeveloped since the start of culture. This proportion is in excellent accord with the Mendelian expectation of 25% Ts homozygotes (X^2 = 0.049).

Karyotype Analysis

Screening of 8 G-banded ideograms, prepared from Ts/+ embryos, failed to reveal any consistent chromosomal deficiences or rearrangements associated with the mutant allele. It was estimated that structural abnormalities constituting 1/7 of the length , or greater, of the medium-sized chromosomes would have been detected with this technique.

Plate 4 Sequence of photomicrographs showing the behaviour, on successive days of culture, of a litter of 10 embryos from a Ts/+ x Ts/+ mating containing 3 presumptive Ts homozygotes.

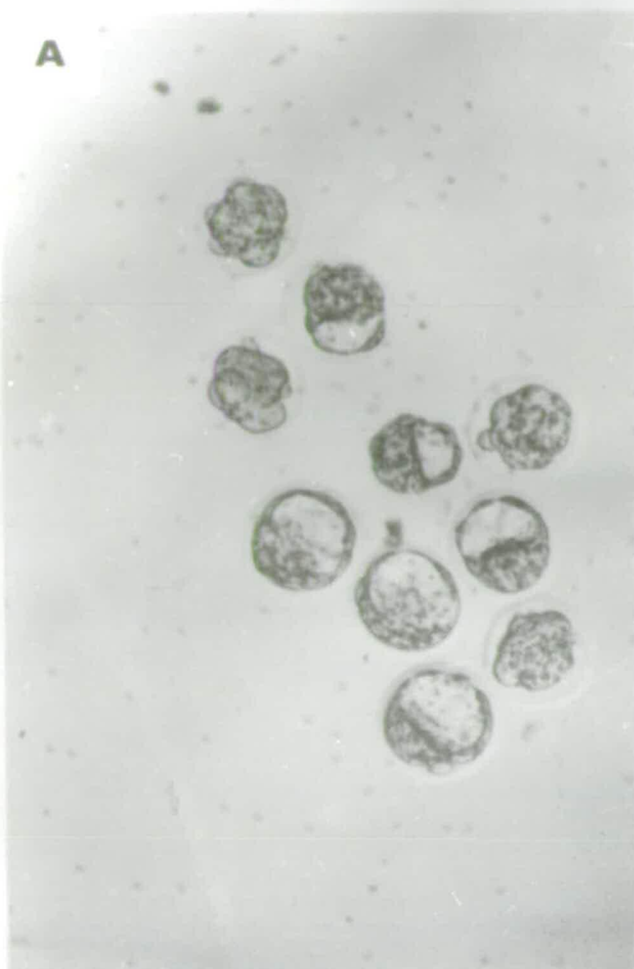
A Start of culture ($3\frac{1}{2}$ days p.c.) . Four embryos have not yet cavitated but appear otherwise normal. One blastocyst is without a zona pellucida.

B 24 hrs culture. Three of those which were previously morulae are still undeveloped (arrowed), and are presumptive Ts/Ts embryos. All but one of the remaining embryos are expanded blastocysts. A zona-free embryo has become attached to the culture dish.

C 48 hrs culture. One Ts/Ts morula is distinctly necrotic. Both the remaining Ts/Ts embryos have developed surface vesicles, and one has a small internal cavity although this is not clearly shown in the photograph. The rest of the litter have now hatched from their zonae, and one is outgrowing.

D 72 hrs culture. Ts/Ts embryos have degenerated into dark necrotic masses. The rest of the litter are out-growing vigorously.

(Magnification x 150)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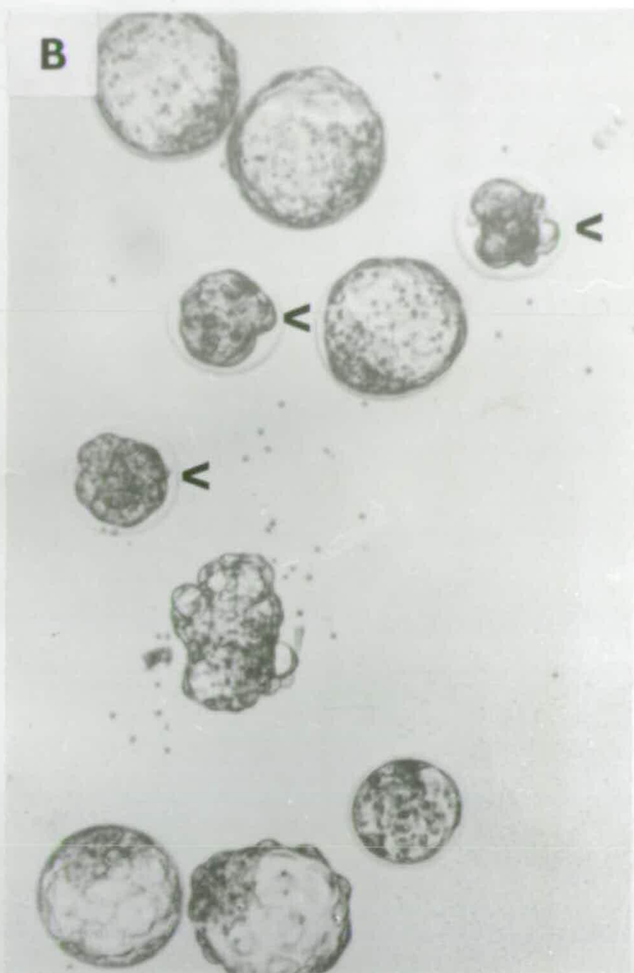
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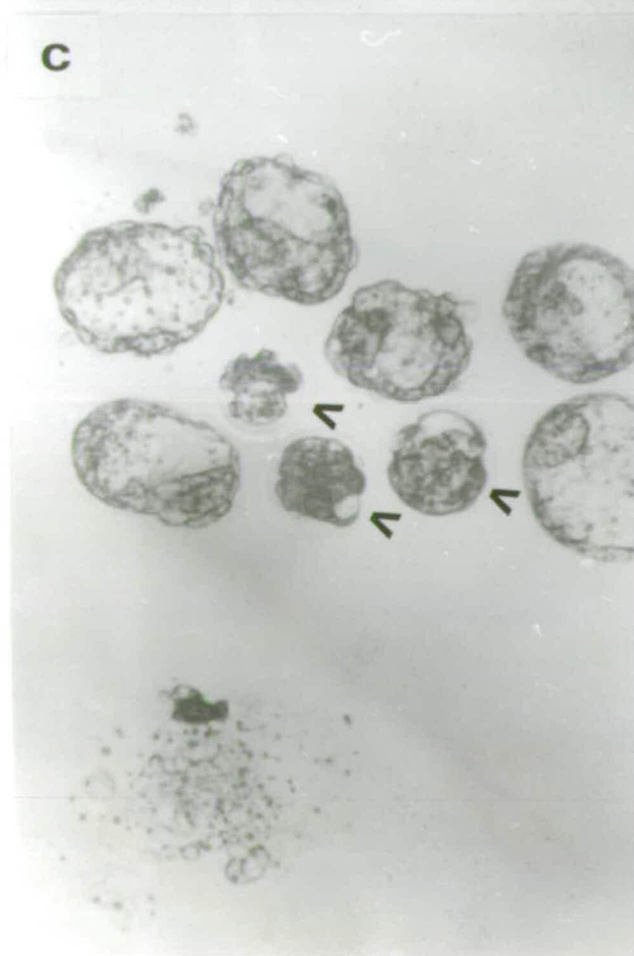
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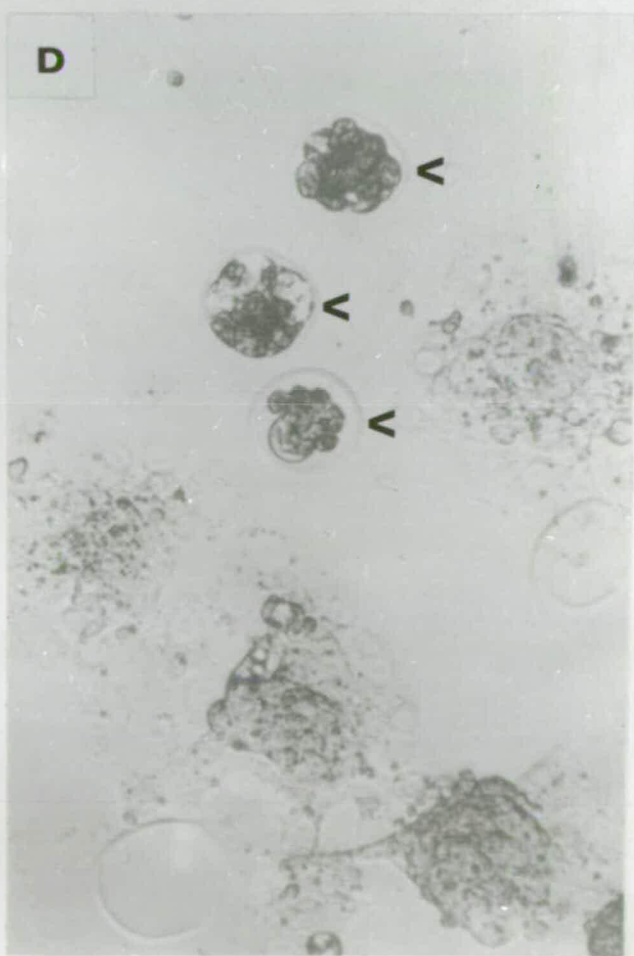
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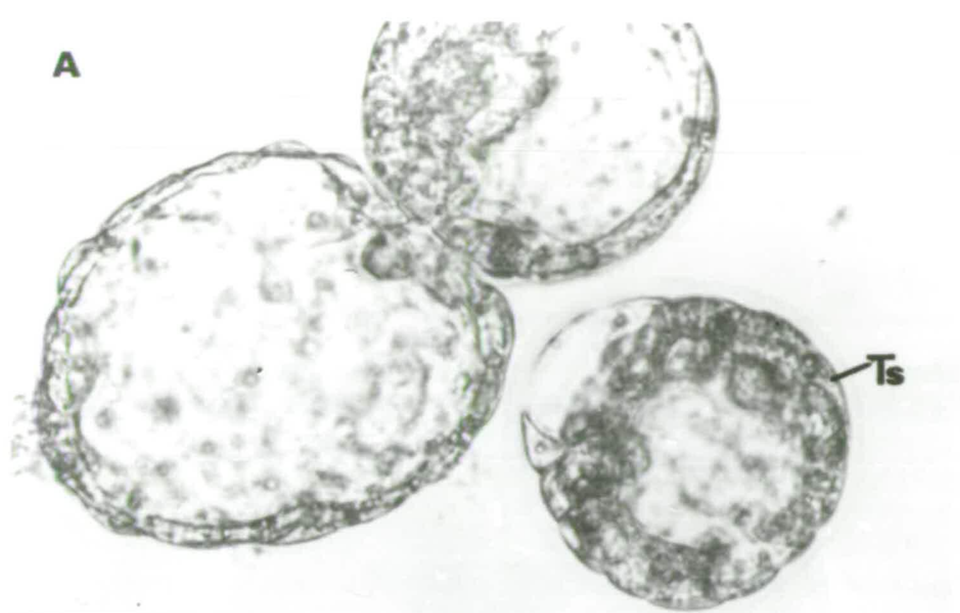
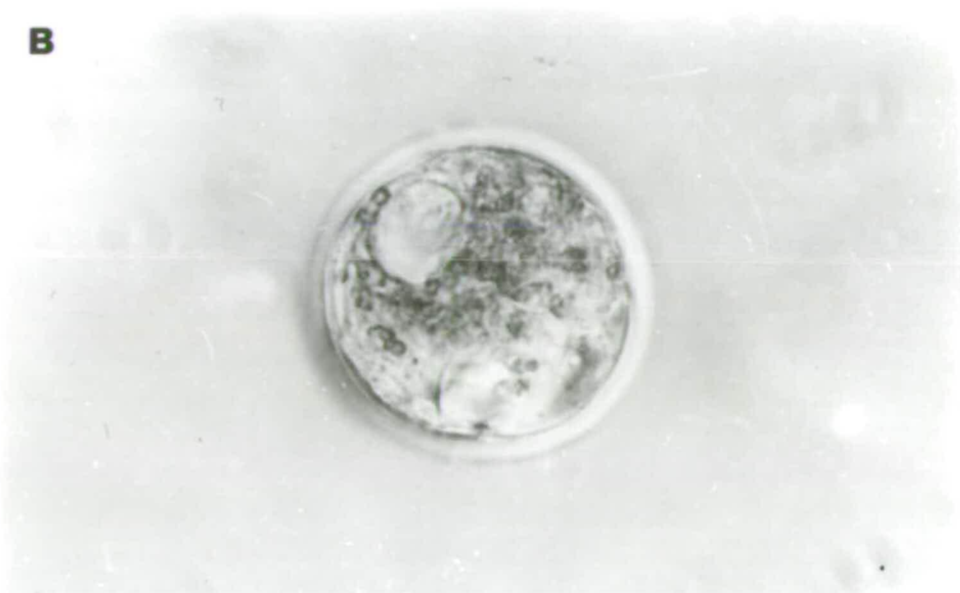
Plate 5 Ts/Ts embryos in culture.

A Presumptive Ts/Ts embryo (Ts) together with hatching littermate after 48 hrs. of culture. The mutant embryo has cavitated but cells are rounded and relatively undifferentiated; note also the surface vesicle.

B Presumptive Ts/Ts embryo after 48 hrs. of culture. A cavity has developed, but morphology is abnormal.

(magnification x 500)


A
Ts


B

DISCUSSION

It is clear from the histology of embryos sectioned *in utero*, in accordance with the conclusions of Deol (1961), that Ts/Ts embryos die at about the time of implantation of their littermates. It is also evident that the great majority of these embryos have not attained a developmental state which is compatible with implantation by this time, and indeed have been visibly retarded for over a day. It seems probable that a few homozygotes start to break up during this period as judged by the partially disintegrated apearance of some of the $4\frac{1}{2}$ day specimens, together with the rather low overall proportion of abnormal embryos present. The fact that the zona pellucida appeared to be still intact in certain cases at $4\frac{1}{2}$ days, suggests that the continued presence of this structure may enable some embryos to maintain their integrity for longer than others. Since escape from the zona is thought to be governed both by a physical hatching mechanism controlled by the embryo itself and by enzymic lysis via the uterus (McLaren 1970; Mintz 1971), absence of the former capability, as may be expected from their retarded state (Noyes and Dickmann 1960) and as is demonstrated in culture, may render the latter process insufficient for completely effective zona removal. It is not possible to say with any degree of certainty whether either or both of the abnormal blastocysts seem to be partially attached to the uterus at $5\frac{1}{2}$ days were Ts homozygotes, for although no such types were found in the controls, a much larger sample of uteri sectioned at this stage would be required for a statistically meaningful inference to be drawn. Certainly if these represent some form of "escaper" Ts/Ts embryos (c.f. A^y/A^y embryos)

then the mutation does not seem to prevent the differentiation of primary invasive cells from the trophoblast. In any case, the presence of occasional decidual swellings which were either empty or contained only a few embryonic remains, strongly indicates that some Ts/Ts embryos manage to survive intact long enough to induce a decidual response.

The pale cytoplasmic staining in haematoxylin and eosin associated with the mutant homozygotes is very similar to that seen in normal embryos up to the 8-cell stage, and therefore presumably represents a failure to effect certain changes in the content of the cytoplasm which should normally occur at this time. Such changes are probably related to the rapid increase in RNA and/or protein synthesis which is known to take place during this period (Mintz 1964a; Monesi and Salfi 196 Ellem and Gwatkin 1968; Woodland and Graham 1969; Daentl and Epstein 1971; Epstein and Smith 1973), and which is clearly demonstrated by the striking increase in cytoplasmic basophilia seen in normally developing embryos stained with Azure B (Alfert 1950; Smith 1956; Mintz 1964c). That RNA synthesis is very much lower in Ts/Ts embryos than in littermates at $3\frac{1}{2}$ days is indicated by their greatly reduced uptake and incorporation of [^{3}H]-uridine, but it cannot be determined from the brief experiment reported here whether this difference is or is not greater than can be accounted for in terms of their respective developmental stages. Of significance is the fact that the low staining capacity of Ts/Ts embryos persists until their final demise, even in those which succeed in cavitating. This feature, then, seems not to be simply a reflection of developmental stage, but presumably results from genuine

metabolic inadequacies associated with the mutation.

The strangely diffuse and ragged nucleoli, noted in some of the presumed homozygous, are of unclear significance since they do not resemble those of normal embryos at any stage in early development. Whilst it is possible that this may be related to peculiarities of RNA metabolism, it cannot be ruled out that it is purely a degenerative change resulting from the distressed state of the embyros. On this basis, those embryos which express this feature most clearly are those which are most rapidly degenerating.

It is evident from the slopes of the curves in Fig. 1 that retardation must begin shortly after the 3rd cleavage, with cell division proceeding at about half the normal rate during the following 24 hours, and subsequently falling off even further in those mutant embryos which survive intact until $4\frac{1}{2}$ days. The small standard error attached to the mean cell number of Ts/Ts embryos at $3\frac{1}{2}$ days indicates that the onset of retardation is quite uniform, and consequently that the variation which occurs with respect to the stage of development reached before death (i.e. morula, blastocyst or even implanting blastocyst) must be determined by independent factors.

The behaviour of Ts/Ts embryos in culture is very similar to the picture presented by the sectioned material _in utero_, although the more static conditions _in vitro_ appear to permit them to survive for about a day longer before degenerating. This prolonged survival may explain why a higher proportion of the mutants succeded in cavitating than was observed _in utero_. It is not surprising that these cavitated embryos, which may best be termed "pseudo-blastocysts", did not

manage to escape from the zona pellucida, for it seems improbable from the data that any had achieved the expanded state which precedes hatching; it may be seen from Table 2 that even the "expanded" blastocysts which ultimately failed to hatch, had become expanded by $4\frac{1}{2}$ days (24 hrs. culture) so that any Ts/Ts embryos reaching this stage would have been remarkably far ahead of their "undeveloped" counterparts. No attempt, in this study, was made to remove the zonae, and it is possible that development might have progressed slightly further if the embryos had been assisted in hatching (c.f. A^y/A^y embryos, Pederson 1974).

It is interesting to compare these observations with the development of embryos homozygous for the t^{12} allele, since they have certain aspects in common. The most obvious similarity between homozygotes for the two mutations is the timing of the phenocritical phases. Both appear abnormal with regard to gross morphology at the morula stage (Smith 1956), although anomalies at the ultrastructural level have been observed as early as the 2-cell stage in presumed t^{12} homozygotes (Hillman et al.1970). Retardation of t^{12}/t^{12} embryos, however, seems to be less severe by $3\frac{1}{2}$ days, at least in terms of cell number, since Smith estimated a mean of 28.4 cells per t^{12}/t^{12} morula _in utero_ at 89 hours, as opposed to an equivalent figure of 13.7 obtained in the present study for Ts/Ts embryos at 88 hrs. _post coitum_. In addition, many Ts/Ts embryos appear to survive longer than t^{12} homozygotes _in utero_, which Smith reported to be, in many cases, small remnant groups of 2-10 isolated cells by 96 hrs. Apparently some t^{12}/t^{12} embryos can form small blastocysts (Hillman et al.1970) but these are

rather rare. Although t^{12}/t^{12} homozygotes at $3\frac{1}{2}$ days stain less densely than normal with Azure B (Smith 1956; Mintz 1964c), it now seems certain that in this case the reduction in cytoplasmic basophilia is a degenerative change caused by solublization of RNA following arrest, and thus is probably a different phenomenon from the pale H & E staining of Ts/Ts embryos. Autoradiography has demonstrated that RNA synthesis, is lower in t^{12}/t^{12} morulae than in their littermates at $3\frac{1}{2}$ days (Mintz 1964a; 1964c), but this also has been shown to result from, rather than cause, the developmental arrest since $[^3H]$-uridine incorporation prior to degeneration is not substantially different from that of normal morulae of the same stage (Erickson et al.1974; Hillman 1972). It may be that the same explanation is true for the low $[^3H]$-uridine uptake and incorporation seen here with Ts/Ts morulae. Aberrant nucleolar morphology is also a characteristic common to both homozygotes, but the two types differ from the norm in a diametrically opposed fashion, with those of t^{12}/t^{12} being abnormally smooth and rounded (Smith 1956) and those of Ts/Ts often being particularly rough and irregular. Yet again, however, this peculiarity of t^{12} homozygotes has been shown to occur only after arrest has taken place and degeneration has commenced (Hillman & Tasca 1969). This type of degenerative change may account for the rounded nucleoli observed in one of the abnormal embryos sectioned *in utero* at $5\frac{1}{2}$ days (Plate 2). Finally, performance in culture, with both types of mutants, more or less mirrors their respective developmental behaviour *in utero*; Mintz (1964b) found no evidence of cavitation in cultured t^{12}/t^{12} embryos. It may be seen, consequently that the similarity between the two recessive lethals is

limited, and is principally restricted to the approximately coincident onset of the phenocritical phases. Paradoxically, the possibility of abnormalities of r-RNA cistrons or nucleolar organiser regions in the DNA reported to be associated with t^{12} (Klein and Raska 1968), but which has subsequently been ruled out as a cause of death in t^{12}/t^{12} homozygotes (see Hillman et al.1970 for discussion), now presents itself as a reasonable explanation of the behaviour of Ts/Ts embryos, since the timing of the phenocritical phase, combined with the persistently pale-staining cytoplasm, abnormal nucleolar morphology, and low [^{3}H]-uridine incorporation is compatible with such a defect.

The chromosomal basis of Ts still remains undetermined, since the karyotype analysis failed to reveal any structural abnormalities. Nevertheless, this in no way implies that the factor is not associated with a deficiency, inversion or translocation, but merely that the extent of the disturbance at the structural level of the DNA is beneath the resolving power of the technique employed. Thus, few inferences can be drawn from this result.

It may be concluded from this initial study that Ts homozygotes die a protracted death spanning the 4th, 5th and in a few cases possibly even the 6th, days of pregnancy. It is uncertain whether or not the mutation interferes with differentiation into trophectoderm and inner cell mass, for although cavitation occurs quite frequently, cells remain relatively undifferentiated in appearance (with the exception of the two $5\frac{1}{2}$ day embryos of indeterminate genotype). E.M. studies will uncover any ultrastructural abnormalities in these embryos and may provide a pointer to the biochemical origin of their developmental inadequacy.

Acknowledgements

I am indebted to Dr. M. S. Deol for introducing this topic, and for providing the experimental animals and a substantial proportion of the histological specimens.

CONCLUSIONS

The three lethal factors which have been examined here reflect some of the earliest directly visible examples of gene expression known in mammalian development. With the relatively simple techniques employed, it was not within the scope of this project to trace the nature of the developmental defects back to their underlying biochemical disturbances . Nevertheless, screening methods for deficiencies or defects in enzymic or structural components within such mutant embryos are potentially available to future investigators, due to the increasingly sensitive electrophoretic and microspectrophotometric techniques presently being pioneered. In order to be certain that observed biochemical anomalies are themselves the source of abnormality rather than mere degenerative changes, it will be desirable to examine the embryos before the lethal phase. Consequently, to be of use with recessive factors which segregate in normal Mendelian ratios , methods must be of sufficient sensitivity either to cope with single embryos, or else be capable of detecting quantitative differences in the metabolic parameters being measured of 25% or less between experimental and control samples. At present, oligosyndactylism seems to be a particularly suitable candidate for this type of approach, as its clearly identifiable intracellular effects, which may be even more precisely examined with the aid of the electron microscope, provide definite clues from which to initiate investigation, at a phenotypic level which is not too far removed from the molecular origin of the lethality. Conversely, tail-short and yellow, as a result of their apparently protracted pre-lethal phases during which development still continues, may enable accurate enough

identification of homozygotes, prior to overall degeneration, to facilitate "harvesting" in adequate numbers for existing biochemical techniques to be applied.

It is hoped that the information obtained in this study may help to provide a basis from which to undertake these types of analyses, and that the investigation of such developmental deficiencies may thus promote an understanding of the immensely complex genetic and environmental interactions which constitute development.

APPENDIX

1. Estimation of Cell Number from Serial Sections

The number of cells in serially sectioned embryos is estimated from counts of nuclear sections according to the formula devised by Abercrombie (1946),

$$p = A \frac{M}{L + M}$$

where p = total number of nuclei (cells) per embryo

A = total number of nuclear sections in serial sections of the entire embryo

M = thickness of sections (μ)

L = mean diameter of nuclei (μ)

2. Estimation of the Frequency of Specific Abnormality

The net frequency of genetically-determined 'specific' abnormality amongst embryos from experimental matings is routinely estimated by subtracting the frequency of abnormality observed in the control group, from the total frequency of abnormality in the experimental group. Where 'non-specific' abnormality is common, however, this method will tend to underestimate the true frequency of 'specific' abnormality since a significant proportion of embryos in the experimental group will be affected by both 'specific' and 'non-specific' factors. Under such circumstances a more accurate determination of the frequency of 'specific' abnormality may be obtained by the use of the following equation:

$$S = \frac{T - N}{1 - N}$$

where S = Net frequency of specific abnormality in the experimental group.

T = total frequency of abnormality in the experimental group

N = frequency of abnormality in the control group

The resultant frequency is thus corrected to include those embryos possessing both categories of abnormalities.

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