Investigation of the lethal yellow $A^y/A^y$ embryo using mouse chimaeras

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SUMMARY

Chimaeric combinations of normal and mutant embryonic tissues were used to investigate the lethal effect of the yellow gene. The homozygous mutant embryos could not be identified before implantation. Therefore, embryos from both intercross matings and control backcross matings were used to provide inner cell masses (ICMs) for injection into genetically marked blastocysts of the CFLP random-bred stock. All conceptuses obtained from reimplanted blastocysts were analysed at mid-gestation for the presence of donor isozyme of glucose phosphate isomerase. A similar proportion of chimaeras were found in the experimental and control series, indicating rescue of the lethal $A^y/A^y$ ICM tissue. The reciprocal experiment also produced a similar proportion of chimaeras but there was a 25% postimplantational loss of injected embryos evidenced by empty decidual swellings. The results suggest that the yellow mutation primarily affects the trophectoderm which cannot be rescued by a normal ICM, whereas $A^y/A^y$ ICM is capable of survival in a chimaera at least until mid-gestation.

INTRODUCTION

Long a favourite of the mouse fancy, the yellow mutant ($A^v$) also has a special place in genetics and developmental biology. It was the first mammalian gene to be identified as a prenatal homozygous lethal because it showed an apparently modified Mendelian ratio among the offspring of phenotypically yellow mice (Cuénot, 1908; Castle & Little, 1910; Ibsen & Steigleder, 1917; Kirkham, 1917, 1919). Using inbred 101$A^v$ mice Robertson (1942) found uniformly abnormal development and death at the end of the fifth day of gestation in 25% of the embryos from heterozygous ($A^v/a$) matings. The abnormal embryos were small blastocysts with no distinguishable endoderm and by the sixth day consisted of only a few scattered cells in uterine crypts in which the epithelium was not eroded normally. By transplanting $A^v/a$ ovaries into $a/a$ females, he was able to extend the life of the $A^v/A^v$ embryos by 1 day. In the $a/a$ uterus, an ectoplacental cone and Reichert's membrane were differentiated and the adjacent uterine epithelium was eroded normally.

Lethality of the homozygous embryos was attributed to a defect in the
trophoblastic giant cells by Eaton & Green (1963) since, although they found that death occurred within a fairly broad time range in the three inbred stocks used, the extent of giant cell differentiation appeared to be the limiting factor. The studies of Pedersen (1974) and Calarco & Pedersen (1976) argue for a much earlier retardation effect in at least some of the cells of the \( A^v/A^v \) embryo leading to the arrest of cleavage. Pedersen (1974) recognized blastomeres arrested from as early as the third cleavage in some embryos and assumed that these were the yellow homozygotes. The presumptive homozygotes failed to hatch from the zona pellucida in vitro and even when the zona was removed artificially, there was no development of inner cell mass and little outgrowth of the trophectoderm. Pedersen concluded that the effects of \( A^v \) occur over a range of time between early cleavage and implantation and are not limited to the trophectoderm.

The primary effect of the mutant and how it brings about the death of the embryo is not clear from these studies. Neither has it been determined to what extent the background genotype of the embryo or mother affects the gene action. \( A^v \) could be a general cell lethal or it could affect the trophectoderm or the inner cell mass (ICM) exclusively, with death of the embryo resulting from the disturbance of normal interactions between these two cell types. We have previously suggested that an interaction between the ICM and trophectoderm is essential for the normal continued proliferation of trophectoderm (Gardner & Papaioannou, 1975); if this interaction were defective in homozygous yellow mice, the embryo would be expected to die.

In the hope of elucidating the events leading to the death of homozygous yellow embryos, we have made use of the fact that tissue from genetically different early embryos can be combined to make chimaeric animals (Gardner, 1968; Gardner, Papaioannou & Barton, 1973). We have combined tissue from normal and mutant yellow embryos at the blastocyst stage and analysed the resulting chimaeric embryos after the usual time of mutant homozygote death. The experiments and some of the possible outcomes are represented schematically in Fig. 1.

**MATERIALS AND METHODS**

*Mice*

The yellow mutant was maintained by forced heterozygosity with extreme non-agouti (\( a^e \)) in the inbred strain AG/Cam (Staats, 1976). This strain is homozygous for the \( Gpi-T^b \) allele at the glucose phosphate isomerase (GPI) locus; GPI was our marker for detecting chimaerism. Females were generally used between 9 and 11 weeks of age and were either mated naturally or after superovulation with 5 i.u. of pregnant mare serum gonadotrophin (Folligon, Intervet) followed 48 h later by 5 i.u. of human chorionic gonadotrophin (Chorulon, Intervet). Heterozygous or intercross matings, \( A^v/a^e \times A^v/a^e \), were used to produce experimental embryos of which 25 % were expected to be
homozygous $A^v/A^v$. To provide the control embryos we used reciprocal backcrosses, $A^v/a^v\times a^v/a^v$ and $a^v/a^v\times A^v/a^v$, in approximately equal numbers. There were no apparent differences between the reciprocal backcrosses so these results have been pooled.

Random-bred albino CFLP females (Anglia Laboratory Animals Ltd.) that were homozygous for the $Gpi-1^a$ allele were superovulated by the same schedule and mated with homozygous $Gpi-1^a$ males to provide the $Gpi-1^a/Gpi-1^a$ embryos for making chimaeras. CFLP or other random-bred females of known $Gpi-1$ genotype were mated to vasectomized males for uterine foster mothers.

**Microsurgery**

Embryos were flushed from the uteri of superovulated pregnant females at 3-5 days post coitum (d p.c.) with PBI medium (Whittingham & Wales, 1969) containing 10% foetal calf serum. Inner cell masses (ICMs) were isolated by dissection with siliconized glass needles using a Leitz micromanipulator (Gardner, 1972), and injected into the blastocoelic cavity of intact host blastocysts using the method of Gardner (1978). The injected embryos were kept at 37 °C for at least 1 h before being transferred to the uteri of 2-5 d p.c. pseudopregnant foster mothers. The embryos were almost always fully re-expanded and contained a single large ICM before transfer. The age of the transferred embryos was subsequently taken as the foster mother’s gestational age. Table 1 gives the $Gpi-1$ genotypes of the ICMs, the host blastocysts and the foster mothers in the two main experiments illustrated in Fig. 1, I and II.

**Analysis of chimaeras**

Conceptuses that developed from injected blastocysts were analysed at 10-5 d p.c. using the GPI isozyme difference to determine the presence of CFLP-derived and AG/Cam-derived tissues. The decidual swellings were removed from the uterus intact and the maternal tissue was dissected away in phosphate buffered saline (PBS). Each conceptus was washed in PBS and then dissected into three samples: the trophoblast comprising the giant cells and the ectoplacental cone, the membranes comprising the yolk sac and amnion, and the foetus. Each sample was washed twice in PBS and frozen in 10 µl distilled
water. It was arranged that the foster mother was the same GPI type as the host blastocyst to avoid confusion by possible contamination of the trophoblast with maternal decidual tissue (Table 1). After thawing, the samples were analysed for GPI by horizontal starch gel electrophoresis (Chapman, Whitten & Ruddle, 1971; Gardner et al. 1973).

Histology

For histological examination, pregnant uteri were fixed in Bouin’s fixative or cold 95% alcohol, wax embedded, sectioned at 8–10 μm and stained with haematoxylin and eosin.

Statistics

All comparisons were tested by the χ² test corrected for continuity (Siegel, 1956) using a significance level of P < 0.05, 1 df. The null hypothesis was tested to determine whether the intercross differed from the backcross and the fit to a 3:1 ratio was tested to determine whether the intercross data fit a 75%:25% segregation. For the latter test the backcross data were used to calculate the expected values.

RESULTS

Histology – natural matings

Since the AG/Cam strain had not previously been used to study the embryonic effects of the yellow mutant, some litters were examined histologically at several gestational ages to establish the time of death of homozygotes and to compare with published work on other strains. At 4.5 d p.c. seven intercross and four control backcross embryos appeared completely normal. At 5.5 d p.c. six backcross embryos were normal but one of six intercross embryos was retarded and had pyknotic nuclei in the trophoderm (Fig. 2). By 6.5 d p.c. there were no living embryonic derivatives, only a few pyknotic nuclei, in 4/18 decidua
Identification of lethal embryos

If $A^v/A^v$ embryos could be recognized at 3-5 d p.c. it would greatly facilitate making lethal-normal chimaeras. Although Pedersen (1974) using C57BL/6J-$A^v/a$ mice was able to distinguish some of the presumptive homozygous embryos at this age by the presence of arrested blastomeres, this was not confirmed in another study using the same strain (Johnson & Granholm, 1978) and it does not seem to be a characteristic of the mutant in our particular inbred strain. We examined a large number of blastocysts from intercross and control backcross matings and found over 30% of all embryos had one or more
Table 2. Incidence of excluded cells in blastocysts from backcross and intercross matings and the incidence of embryonic death of transferred embryos with and without excluded cells

<table>
<thead>
<tr>
<th>No. of blastocysts with excluded cells/Total</th>
<th>Embryonic death of transferred embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of blastocysts</td>
<td>%</td>
</tr>
<tr>
<td>Backcross</td>
<td>46/143</td>
</tr>
<tr>
<td>Intercross</td>
<td>63/171</td>
</tr>
</tbody>
</table>

excluded cells. When embryos were sorted, transferred to foster mothers and examined 3–4 days later, there was no indication that the groups of intercross embryos with excluded cells contained any more $A^v/A^v$ embryos than those without (Table 2). It is possible that the excluded cells we found are actually persisting polar bodies as described by Lewis & Wright (1935). In several other more limited series we transferred embryos that had been sorted on the basis of size, developmental stage or other morphological criteria but could find no single identifying characteristic of homozygous $A^v/A^v$ embryos in AG/Cam mice at 3-5 d.p.c.

Since the embryos for microsurgery were all from superovulated mothers and only the well expanded ‘operable’ blastocysts were used, it was important to ensure that this selection procedure did not in fact exclude the $A^v/A^v$ embryos. We transferred a number of embryos selected as suitable for microsurgery to foster mothers and examined them histologically at 6-5 d.p.c. (Table 3). From the operable backcross embryos transferred, 5% of the decidual swellings contained no embryonic derivatives by 6-5 d.p.c., whereas this figure was 32% for the intercross embryos, a difference of just over 25%, indicating that the homozygous $A^v/A^v$ embryos are among the normal looking, well expanded blastocysts from superovulated mothers and therefore among the embryos suitable for microsurgery.

**ICM injections**

ICMs were successfully dissected from about 95% of all AG/Cam embryos attempted with no difference between the reciprocal backcrosses and the intercross. The efficiency of injection of AG/Cam ICMs into CFLP embryos was also high, about 95%, and again there was no difference between backcross and intercross embryos. When CFLP ICMs were injected into AG/Cam embryos the efficiency of injection was 89% for backcross embryos and 86% for intercross embryos, not a significant difference.

AG/Cam embryos were used in the two different experiments as either the
Lethal yellow $A^y/A^y$ mouse embryos

Table 3. Examination at 6·5 d.p.c. of transferred 'operative' blastocysts from superovulated females

<table>
<thead>
<tr>
<th>No. of foster mothers*</th>
<th>No. of deciduae/No. of embryos transferred</th>
<th>No. of embryos</th>
<th>Yolk sac or giant cells only</th>
<th>Without embryonic derivatives</th>
</tr>
</thead>
<tbody>
<tr>
<td>Backcross</td>
<td>14</td>
<td>76/110 (69%)</td>
<td>70</td>
<td>2</td>
</tr>
<tr>
<td>Intercross</td>
<td>14</td>
<td>92/103 (89%)</td>
<td>61</td>
<td>2</td>
</tr>
</tbody>
</table>

* Excluding those that failed to become pregnant.

Table 4. AG/Cam ICMs injected into CFLP blastocysts: implantation rates and proportions of chimaeric conceptuses at 10·5 d.p.c.

<table>
<thead>
<tr>
<th>ICM</th>
<th>Intercross</th>
<th>Backcross</th>
</tr>
</thead>
<tbody>
<tr>
<td>Implantation rate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of decidual swellings/No. of embryos transferred</td>
<td>54*/62 = 87%</td>
<td>70†/80 = 88%</td>
</tr>
<tr>
<td>Proportion of chimaeras</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total no. of chimaeras/Total no. of conceptuses</td>
<td>44‡/53 = 83%</td>
<td>62§/66‖ = 94%</td>
</tr>
</tbody>
</table>

* One swelling contained membranes only that were not successfully typed for GPI.
† One decidual swelling lacked embryonic derivatives.
‡ Including one retarded chimaera and two chimaeras with twin yolk sacs (see Discussion).
§ Including three retarded chimaeras and three chimaeras with smaller twin embryos.
‖ In addition there were three decidual swellings containing only trophoblast and membranes, two were chimaeric.

host blastocyst or the ICM donor. These reciprocal experiments will have different results depending on the site of action of the yellow gene, but should be complementary in the information they provide as outlined in Fig. 1. Since $A^u/A^u$ blastocysts could not be recognized, we were forced to use all the embryos from the intercross of which only 25% will be $A^u/A^u$. The reciprocal backcross injections gave us control levels of implantation, chimaerism and embryonic death to compare with the intercross in assessing the behaviour of $A^u/A^u$ tissue.

Table 4 shows the results of injecting AG/Cam ICMs into CFLP blastocysts (Fig. 1, I). The implantation rate of injected blastocysts was similar for both intercross and backcross, and only one decidual swelling lacked embryonic derivatives, indicating that the presence of a lethal $A^u/A^u$ ICM is not detrimental to implantation of the chimaeric blastocysts. With this established, we then want to know whether the proportion of chimaeric embryos is the same or
Table 5. CFLP ICMs injected into AG/Cam blastocysts: implantation rates and proportions of chimaeric conceptuses at 10.5 d.p.c.

<table>
<thead>
<tr>
<th></th>
<th>Host blastocyst</th>
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<tbody>
<tr>
<td></td>
<td>Intercross</td>
<td>Backcross</td>
</tr>
<tr>
<td>Implantation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of decidual swellings/No. of embryos transferred</td>
<td>73/99 = 74%</td>
<td>89/98 = 91%</td>
</tr>
<tr>
<td>No. of swellings with embryonic derivatives/No. of swellings</td>
<td>58/73 = 79%</td>
<td>87/89 = 97%</td>
</tr>
<tr>
<td>Proportion of chimaeras</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total no. of chimaeras/Total no. of conceptuses</td>
<td>47*/55† = 85%</td>
<td>68‡/82 = 83%</td>
</tr>
</tbody>
</table>

* Including four recently dead chimaeric embryos and three chimaeras with a smaller twin embryo or yolk sac.
† Including one retarded and one slightly deformed non-chimaeric embryo. In addition there were three decidual swellings containing only giant cells and membranes, two of which were chimaeric.
‡ Including one retarded, four recently dead, two slightly deformed and one twin chimaeras. In addition there were five decidual swellings containing only giant cells, one chimaeric.

25% lower in the intercross injected embryos. Using the rate of chimaerism of the reciprocal backcross as the control level, the rate of chimaerism in the intercross is significantly different from the 25% decrease expected if $A^v/A^v$ ICM tissue dies ($\chi^2 = 4.02, P < 0.05$). The slightly lower rate of chimaerism in the intercross could indicate some disadvantage of the $A^v/A^v$ ICM tissue, however, this rate of chimaerism is not significantly different from the control ($\chi^2 = 2.6, P > 0.10$).

The observed results of the injections of CFLP ICMs into AG/Cam blastocysts are shown in Table 5 and can be compared with the possible expected results outlined in Fig. 1, II. Although the proportion of chimaeras is not significantly different between the intercross and backcross, there are significant differences in both the implantation rate of injected embryos and in the proportion of decidual swellings containing embryonic derivatives. In both cases the differences are not significantly different from a 25% decrease in the intercross compared to the backcross ($\chi^2 = 1.4, P > 0.20$).

**Discussion**

The results of the injection experiments shown in Tables 4 and 5 can be interpreted by comparison with the expected results outlined in Fig. 1. The microsurgical combinations of CFLP tissue with intercross AG/Cam ICMs or blastocysts (genotypes $A^v/A^v$, $A^v/a^e$ and $a^e/a^e$) are compared with similar combinations involving backcross AG/Cam tissue (genotypes $A^v/a^e$ and $a^e/a^e$).
The interpretation of the results will depend on whether the involvement of lethal $A^v/A^v$ tissue in a blastocyst chimaera results in a lower implantation rate, lower embryonic survival, or a lower proportion of chimaeric conceptuses later in development, or whether these are all similar in the two series indicating a rescue of the lethal tissue.

In all experiments chimaerism was prevalent in the foetus and membranes but often with low levels in the trophoblast fraction (possibly in the parietal endoderm or allantois since these were included with the trophectoderm). In both the backcross and the intercross there were a few conceptuses with chimaerism only in the membranes or foetus. Also each series had a small number of abnormal conceptuses such as occasional decidual swellings with only a few giant cells and possibly membranes, or, more interestingly, ‘twin’ embryos (see footnotes to Tables 4 and 5). These twins usually consisted of a normal chimaeric embryo with a second yolk sac which sometimes contained a retarded foetus, all within the same trophoblastic shell. Since the larger of the two, and usually both, were chimaeric, these twins are probably the result of incomplete incorporation of the injected inner cell mass with some of the unincorporated cells attaching to a second site on the inner surface of the trophectoderm. Overall the rate of twinning was nearly 4%.

$A^v/A^v$ ICMs into normal blastocysts

The fact that the rates of chimaerism between the backcross and intercross are not significantly different indicates that $A^v/A^v$ ICM cells are capable of growth and survival up to at least 10-5 d p.c. and therefore that the death of intact $A^v/A^v$ embryos is not attributable primarily to an ICM defect. The results also suggest that the $A^v$ mutant is not a general cell lethal. They are compatible with a trophectoderm defect, since $A^v/A^v$ trophectoderm was not involved in this experiment, or with a defect in a trophectoderm–ICM interaction since the presence of both normal components in the host blastocyst could have provided any necessary interaction for the lethal ICM.

Normal ICMs into $A^v/A^v$ blastocysts

The second series of injections of CFLP tissue into AG/Cam blastocysts was designed to further characterize the mutant effect by testing the capacity of $A^v/A^v$ trophectoderm to support the development of chimaeric embryos. In this series the proportion of chimaeras was the same in the backcross and the intercross. However, there was a reduction in the proportion of surviving embryos compatible with the idea that 25% of the intercross embryos die soon after decidualization at a time similar to the time of death of non-chimaeric $A^v/A^v$ embryos.

It should also be pointed out that the implantation rate of the intercross embryos is significantly lower than the backcross. However, since preimplantation loss (i.e. before decidualization) is not a characteristic of the $A^v$
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mutant and since all the injected embryos appeared to be fully recovered from the injection procedure before they were transferred to foster mothers, we prefer to think that the different implantation rates are fortuitous and that the postimplantation loss represents the loss of \( A^v/A^v \) embryos.

CONCLUSIONS

The combined results of the two injection experiments have provided strong indications that the lethal yellow gene mainly affects the trophectoderm and that the \( A^v/A^v \) ICM tissue can survive to at least 10-5 d.p.c. in combination with normal tissue. We have found no evidence of \( A^v \) acting as a general cell lethal or exclusively affecting the ICM or ICM–trophectoderm interactions. The fact that the \( A^v/A^v \) embryos are indistinguishable from others at the blastocyst stage in AG/Cam mice, however, has made reliance on statistical differences necessary and therefore the results must be interpreted somewhat cautiously. The probability of rescuing \( A^v/A^v \) trophectoderm with normal ICM tissue, for example, may be dependent on the extent of incorporation of the injected ICM which, from the number of ‘twins’ found, we can assume to be variable. The incorporation of normal ICM tissue in sufficient amount and in the correct position to effect rescue may occur in such a small proportion of the 25 % \( A^v/A^v \) embryos that we could not distinguish it by statistical means.

The in vitro culture of isolated ICMs in preliminary experiments has so far corroborated our present interpretation of a trophectoderm defect since there does not appear to be a 25 % decrease in the survival of ICMs from intercross matings (Papaioannou, unpublished). The technically more difficult but more easily interpreted experiment of embryo reconstitution (Gardner et al. 1973) using trophectoderm from one embryo and ICM from another is being attempted with the lethal yellow embryo to further clarify the effect of the yellow gene.

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**Lethal yellow A\textsuperscript{v}/A\textsuperscript{v} mouse embryos**


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