Intrinsic and extrinsic factors affecting the viability of *Mus caroli* × *M. musculus* hybrid embryos*

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Summary. Interspecific hybrids between *M. musculus* and *M. caroli*, a wild species of mouse, were produced by artificial insemination, although the species do not normally interbreed. However, the success rate was low, with many embryos dying at various stages of pregnancy. Hybrid embryos were retarded in comparison with either parent species from the earliest stages of development, suggesting that intrinsic problems of genomic incompatibility play a major role in poor hybrid survival. However, failure of normal embryo–uterine interactions may also be important, since *M. caroli* × *M. caroli* embryos transferred to the *M. musculus* uterus also failed to survive to term. It is suggested that a maternal immune response to antigens on the foreign trophoblast may be involved.

Introduction

Production of interspecific hybrids between *Mus musculus* and wild species of *Mus* has great potential as a means of introducing new genetic variants into the gene pool of laboratory mice. In particular, variants of X-linked enzyme loci would be very useful additions to the gene pool, facilitating studies of X-inactivation in early embryos. *M. caroli*, a wild species from S. E. Asia (Marshall, 1972) carries genes for electrophoretic variants of 3 X-linked enzymes (Chapman & Shows, 1976). *M. caroli* and *M. musculus* do not interbreed naturally but some success has been reported in producing viable, but so far infertile, hybrids by artificial insemination of *M. musculus* with spermatozoa from *M. caroli* (West, Frels, Papaioannou, Karr & Chapman, 1977; West, Frels & Chapman, 1978). However, progress in this direction has been hampered by the low and variable rate of successful hybrid development. Many hybrid embryos die at various stages of pre- and post-implantation development and few survive to term. The aim of this paper was to investigate whether factors other than internal genomic incompatibility could play a role in causing this embryo death.

Poor hybrid survival might result if the embryonic development of the two species were grossly different. Hybrid cells might be subjected to conflicting morphogenetic signals, impairing normal embryonic growth. The embryological development of *M. caroli* has not been recorded and so we have undertaken a comparative study of the pre- and post-implantation development of *M. caroli* and *M. musculus* to see whether major differences do occur. Hybrid survival might also be impaired if the expression of *M. caroli* genes in the *M. musculus* uterus interferes with normal embryo–uterine interactions. In particular, the interaction between trophoblast and uterus at implantation appears to be critical for embryo survival and to be very specific. Various

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studies on interspecific embryo transfer, particularly of rat embryos to the mouse uterus, have shown that such embryos usually die around implantation, apparently because of a failure of this trophoblast–uterine interaction (Tarkowski, 1962; Copp & Rossant, 1978; Tachi & Tachi, 1979). Hybrid embryos might also suffer similar problems. Even if some embryos can become established at implantation, the presence of foreign species antigens may later provoke an immune response in the mother leading to embryo destruction. In normal intraspecific pregnancies such rejection does not occur (reviewed by Beer & Billingham, 1971), despite the presence of paternal antigens on the fetus, but some suggestion of an anti-species response has been reported in female mice carrying rat–mouse chimaeras (Gardner & Johnson, 1975). In the present study, interspecific transfer of *M. caroli* embryos to *M. musculus* uteri was performed to investigate whether normal embryo–uterine interactions can occur between these two fairly closely related species or whether failure of such interactions could help to explain hybrid embryo death.

Materials and Methods

Random-bred HA(ICR) mice (Roswell Park Production Facility, West Seneca, New York) were used throughout to represent the species *Mus musculus*. *Mus caroli* were maintained from a stock originally provided by Dr J. T. Marshall, Bangkok. Preimplantation embryos were obtained from females of both species after induction of superovulation with PMSG (Gestyl: Organon) and hCG (Sigma). Hybrid embryos were obtained by artificial insemination of *M. musculus* females with spermatozoa from *M. caroli* males as described previously (West *et al.*, 1977). All three types of pre-implantation embryo were flushed from the oviducts or uteri, at various stages after hCG injection, by using PB1 medium + 10% fetal calf serum (Whittingham & Wales, 1969). Embryos were examined under the dissecting microscope and any grossly abnormal ones were discarded. Cell counts were performed on all the rest, using Tarkowski's air-drying method (Tarkowski, 1966).

Post-implantation embryos from *M. caroli* × *M. caroli* natural matings were examined histologically. Females were killed on the afternoon of the 6th, 7th and 8th day after mating, and any decidual swellings were fixed in formol acetic alcohol, dehydrated and embedded in paraffin wax. Sections were cut at 7 µm and stained with haemalum and eosin. Later embryos were dissected from the uterus, examined intact under the dissecting microscope and staged by comparison with *M. musculus* embryonic development (Theiler, 1972).

M. caroli embryos for transfer were flushed from the uterus between 76 and 82 h after hCG, when most were at the blastocyst stage. *M. musculus* females were mated with vasectomized males and *M. caroli* blastocysts were transferred into their uteri on the 3rd day of pseudopregnancy. In some cases, *M. musculus* blastocysts were transferred into the contralateral horn. Early post-implantation development was followed histologically and later development by direct examination after dissection. Many recipients were allowed to proceed to term. If no offspring were born, the recipients were then killed and examined for resorptions.

Results

Comparison of preimplantation development of M. caroli, M. musculus and hybrids

Cell counts and observations on preimplantation stages revealed that *M. caroli* embryos pass through preimplantation development much faster than *M. musculus* (Table 1). This is not due to early mating or fertilization since pronuclear formation occurs at about the same time in both species (unpublished data). Until 73 h after hCG the cleavage of *M. caroli* embryos was much faster than that of *M. musculus* embryos but the rates of increase then appeared similar. This

apparent similarity is almost certainly an artefact because by 85 h after hCG when *M. musculus* embryos were 19-cell morulae, *M. caroli* embryos had already lost their zonae and were starting to implant. This means that cell numbers for *M. caroli* at 85 and 100 h were underestimates, since the embryos were difficult to recover intact and cell spreads were not so readily made. *M. caroli* embryos reached the blastocyst stage around 76–80 h after hCG, whereas *M. musculus* embryos were not blastocysts until 92–96 h after hCG. By implantation, *M. caroli* embryos were approximately 16–20 h ahead of *M. musculus* in development. The gestation period of *M. caroli* is about 2 days shorter than *M. musculus* and it seems that a large part of this difference is brought about by faster preimplantation development.

Table 1. Cell counts (mean ± s.e.m.) on *M. caroli*, *M. musculus* and hybrid embryos during preimplantation development

	Time after hCG (h)					
	41	48	53	73	85	100
<i>M. caroli</i> × <i>M. caroli</i>	2.5 ± 0.2 (16)	3.9 ± 0.2 (53)	4.3 ± 0.4 (19)	31.3 ± 1.4 (40)	49 (1)*	99.4 ± 3.0 (7)*
<i>M. musculus</i> × <i>M. musculus</i>	1.8 ± 0.1 (66)	2.0 ± 0.0 (40)	2.6 ± 0.1 (82)	10.6 ± 0.3 (107)	19.8 ± 1.0 (33)	68.5 ± 2.8 (20)
<i>M. caroli</i> × <i>M. musculus</i>	1.6 ± 0.1 (82)	1.7 ± 0.0 (158)	1.8 ± 0.1 (88)	5.8 ± 0.3 (31)	9.8 ± 0.8 (21)	28.4 ± 1.9 (47)

* Small numbers because blastocysts already attached to uterus.

The cell numbers in the hybrid embryos were not intermediate between *M. caroli* and *M. musculus* but consistently fell below either of the two parent species. These cell counts excluded the fairly large number of grossly abnormal or uncleaved embryos. Hybrid embryos therefore appeared retarded from the beginning of development, and this could have interfered with their chances of post-implantation survival. Such retardation has been briefly noted before (West *et al.*, 1977).

Postimplantation development of *M. caroli*

Ten 5½-day *M. caroli* embryos from 3 mice were sectioned and examined. All were late egg cylinders similar to those of *M. musculus* at 6½ days. Morphologically, the embryos were of two distinct types. The first closely resembled a *M. musculus* egg cylinder: the embryonic and extraembryonic ectoderm were thick and columnar and the proamniotic cavity was fairly small. The other type of embryo (Pl. 1, Fig. 1) had a much enlarged proamniotic and extraembryonic cavity. The ectoderm layer was thinner, as though stretched by the expansion of the internal cavity, which often extended almost into the ectoplacental cone. We have not observed this type of structure in *M. musculus* but it is not clear whether all embryos pass through this stage or whether only some have an expanded cavity. Only one embryo showed traces of mesoderm formation.

Eight 6½-day *M. caroli* embryos from 3 mice were sectioned and all were morphologically very similar to 7½–8-day *M. musculus* embryos (Pl. 1, Fig. 2). In all cases, the amnion was complete and the allantois was forming, but the chorionic ectoderm was not yet fused to the ectoplacental cone. Four 7½-day *M. caroli* embryos were sectioned and all were at early somite stages with neural groove, dorsal aortae, fused allantois and chorion, etc. Embryos dissected from the uterus at later stages were all morphologically indistinguishable from *M. musculus* but were always 1½–2 days ahead of equivalent *M. musculus* stages.

Development of *M. caroli* embryos in *M. musculus* uteri

A total of 195 *M. caroli* blastocysts were transferred to 29 *M. musculus* recipients, of which 23 (containing 155 embryos) became pregnant. From these recipients, 29 *M. caroli* fetuses were obtained, 18.7% of those transferred. This rate of successful development includes fetuses analysed at all stages of development up to and including term. The breakdown of the figures for the different days of development is given in Table 2, where it can be seen that the rate of survival falls as gestation proceeds. Recipients analysed between 5½ and 11½ days of pregnancy contained 21 normal embryos out of 49 transferred (42.9%) whereas recipients analysed between 13½ and 18½ days contained only 7 embryos out of 37 transferred (18.9%). The rates of implantation for both periods were much higher (65.3 and 78.4% respectively), showing that considerable embryo death occurred by resorption fairly late in pregnancy. The number of embryos that survived to term was very low: 1 out of 69 transferred (1.5%). This one fetus was not nursed and died 1 day after birth. Nearly all recipients which were allowed to go to term appeared pregnant up to around 12 days, and then resorption must have occurred.

Table 2. Development of *M. caroli* embryos in *M. musculus* uteri

Embryos	Day of development								
	5½	6½	9½	10½	11½	13½	15½	16½	18½
No. transferred	10	18	8	2	11	6	11	10	10
No. implanted	10	11	8	0	3	4	7	8	10
No. surviving	10	7	4	0	0	2	3	2	0

Morphologically, nearly all embryos recovered were normal. Early post-implantation stages (5½ and 6½ days) were sectioned and appeared similar to *M. musculus* embryos of the same age (Pl. 1, Figs 3 and 4). A few 6½-day embryos showed the beginning of primitive streak formation. Later embryos were also very similar to control *M. musculus* transfers, showing again that most of the asynchrony between *M. caroli* and *M. musculus* occurs before implantation. The rate of successful development varied considerably from recipient to recipient and could not necessarily be correlated with successful development of *M. musculus* embryos in the other horn. One marked example of this occurred in one recipient which contained 3 normal 15½–16 day *M. musculus* embryos in one horn and 3 dead, 12–14½ day *M. caroli* embryos in the other horn. This was the only recipient in which such dead fetuses were actually observed, but death followed by resorption must have occurred in many other recipients as well.

Discussion

The present study has shown that the poor survival of *M. caroli* × *M. musculus* hybrid embryos can be attributed mostly to intrinsic problems of genomic incompatibility but that the chances of hybrid survival may be further reduced by failure of normal embryo–uterine interactions. Comparison of the embryonic development of *M. caroli* and *M. musculus* revealed no major differences except in timing of the different stages. *M. caroli* embryos cleave much faster than

PLATE 1

- Fig. 1. Section of 5½-day *M. caroli* egg cylinder with expanded proamniotic cavity. Bar = 50 µm.
- Fig. 2. Section of 6½-day *M. caroli* conceptus. Bar = 100 µm.
- Fig. 3. Section of *M. caroli* embryo in *M. musculus* uterus at 5½-days of development. Bar = 50 µm.
- Fig. 4. Section of *M. caroli* embryo in *M. musculus* uterus at 6½-days of development. Bar = 50 µm.

PLATE 1

(Facing p. 390)

M. musculus and implant 16–20 h earlier. They may also undergo post-implantation development a little faster but the difference is not so dramatic. One might expect that hybrid embryos could adjust for this difference in developmental rate and divide at a rate somewhere between that of the two parent species. In fact, the cell numbers of the hybrid embryos are lower than those of either parent species throughout pre-implantation development and the proportion of abnormal cleavage stages is very high (West *et al.*, 1977). This indicates that there are intrinsic difficulties in the formation of *M. caroli* × *M. musculus* hybrids which cause retarded or abnormal development from the earliest stages. If embryos are retarded at the normal time of implantation, this could also explain why post-implantation development is poor. However, transfer of 3½-day hybrid embryos to 2½-day pseudopregnant recipients does not increase survival (West *et al.*, 1977), suggesting that other factors may be involved in post-implantation death.

One such factor may be the abnormal interaction of uterus and embryo, because the overall survival of *M. caroli* embryos transferred to the *M. musculus* uterus is no better than the survival of hybrid embryos. Unlike rat embryos transferred to the mouse uterus (Copp & Rossant, 1978), *M. caroli* embryos apparently undergo implantation and early post-implantation development normally in *M. musculus*, but considerable embryo death does occur at later stages and survival to term is rare. The mean survival time of the embryos seems to vary from mother to mother, as has been noted in hybrid development (unpublished observations). These two observations suggest that a maternal immune response to foreign species antigens may be involved in late embryo death in both interspecific transfers and hybrids. Such a response would take time to develop and is likely to vary between individual mothers, since random-bred stocks were used. This possibility will be investigated further by testing for humoral and cellular immunity to *M. caroli* antigens in *M. musculus*. Whatever the cause of death in interspecific transfers, the trophoblast must be the tissue that is causing the problems, since interspecific chimaeras made by injecting *M. caroli* inner cell masses into *M. musculus* blastocysts survive to term at a high frequency in a *M. musculus* uterus (Rossant & Frels, 1980). Further investigation of this interspecific transfer system may, therefore, help to evaluate the importance of trophoblast–uterine interactions for embryo survival at various stages of pregnancy.

Given the intrinsic problems of hybrids as well as external uterine effects, it is perhaps not surprising that the rate of hybrid development is so low. Three possible methods of improving hybrid success are suggested by the present study. Firstly, if immune rejection of hybrid fetuses is a major factor in causing embryo death, one might attempt to make hybrids using athymic nude *M. musculus* mothers, which cannot produce an immune response (Pantelouris & Hair, 1970). Secondly, viable hybrid blastocysts obtained from other *M. musculus* strains inseminated with *M. caroli* spermatozoa could be transferred in nude mice. Thirdly, inner cell masses from hybrid blastocysts could be injected into *M. musculus* trophectoderm vesicles which would protect the hybrid cells in the uterus.

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