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Interspecific Hybrids and Chimeras in Mice

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ABSTRACT Interspecific hybrids and chimeras in mammals provide unique tools for investigating problems in genetics and embryology, because of the degree of disparity between the two component genotypes. We have attempted to produce hybrids and chimeras between *Mus musculus*, the laboratory mouse, and *Mus caroli*, a wild species of mouse from Southeast Asia. *M. musculus* and *M. caroli* do not normally interbreed, although sterile hybrids can be produced at a low rate by artificial insemination. Extrinsic problems of genotypic incompatibility between the fetus and the maternal environment seem to be involved in poor hybrid survival, since *M. caroli* blastocysts also die when transferred to the *M. musculus* uterus. Death is associated with the generation of maternal T-cells which are cytotoxic to *M. caroli* target cells in vitro. It is not yet clear whether this immune response is the primary cause of death or is secondary to breakdown of some other components of the fetal-maternal interaction. It is clear, however, that it is the trophoblast layer that mediates survival or death of the foreign embryonic cells in the *M. musculus* juterus, since *M. caroli* inner cell mass cells can survive to term after injection into *M. musculus* blastocysts: Viable interspecific chimeras result. Even more convincing evidence is provided by the production of viable *M. caroli* offspring by trophoblast vesicle reconstitution using trophoblast of *M. musculus* genotype and inner-cell mass of *M. caroli* type. Studies of properties of isolated trophoblast tissues have indicated that *M. caroli* trophoblast may differ from *M. musculus* in both its antigenic and immunosuppressive properties. Elucidation of trophoblast-uterine interactions in these various interspecific pregnancies is being aided by the development of an in situ marker system, which can distinguish cells of the two species in sectioned material by in situ hybridization with a *M. musculus* satellite DNA probe. This same marker is also proving a very powerful tool for analyzing cell lineage development in chimeras. *Key words* interspecific chimeras, interspecific hybrids, trophoblast, *Mus caroli*, trophoblast-uterine interactions, maternal immune responses in pregnancy

Pregnancies between different species of mammals introduce a degree of genetic disparity, either within the embryo or between the embryo and the mother, that can be exploited to address various problems in developmental biology. As will be discussed by Kraemer ('83), interspecific pregnancies produced by either hybridization or embryo transfer are rarely successful. Many explanations have been proffered to explain the failure of such pregnancies (Chang and Hancock, '67; Beer and Billingham, '76), but there has been limited experimental analysis in most systems. We have recently decribed an interspecific pregnancy system in rodents in which a combination of genetic and embryological manipulation has allowed us to define more clearly the parameters of success or failure in pregnancies between species (Frels et al., '80; Rossant et al., '82). This system involves various pregnancies between two species of mice—*Mus musculus* and *Mus caroli*, a wild species of mouse from Southeast Asia (Marshall, '77), which has been maintained as a laboratory stock for several years. The two species are genetically distinct and do not normally interbreed (West et al., '77). In this paper, we describe the rationale for seeking to establish successful pregnancies between these two species and detail some of

the information that such pregnancies have provided about embryonic development and fetal-maternal interactions in mammals.

WHY CROSS THE SPECIES BARRIER?

We have attempted to produce two different kinds of animals in which the genotypes of *M. musculus* and *M. caroli* coexist—interspecific hybrids and chimeras. In both cases, known genetic differences between the two species suggested that such animals would provide very useful research tools. It was originally hoped that interspecific hybrids would provide a means of introducing new genetic variants, particularly of X-linked genes (Chapman and Shows, '76), found in *M. caroli*, into the gene pool of *M. musculus*. Even if hybrids proved to be sterile, as is commonly found in interspecific crosses (Haldane, '22), the ability to bring the genes of the two species together in the same cell would in itself be useful. For example, several of the alleles of X-linked genes found in *M. caroli* differ from the corresponding alleles in *M. musculus* (Chapman and Shows, '76; West et al., '77) in the electrophoretic properties of their corresponding proteins, thus making interspecific hybrid embryos and cell lines very useful tools for studying the process of X-chromosome inactivation (West et al., '78; Marshall-Graves, '82). Also, kidney β-glucuronidase and several other androgen-inducible genes in *M. musculus* are not induced by androgen in *M. caroli* (R. Swank et al., unpublished), suggesting that hybrids will provide useful insight into the genetic mechanism of androgen inducibility.

Interspecific chimeras, on the other hand, would not allow examination of the two genotypes in the same cell, but would provide a useful system for studying the distribution of cells of the two genotypes during development. Genetic chimeras, which are made by mixing cells of two different genotypes early in development (Mintz, '62; Tarkowski, '61; Gardner, '68), have proved very useful for studying cell lineage and cell determination during development (Mintz, '74; McLaren, '76; Rossant, '83). However, detailed clonal analysis of cell distribution in such chimeras has been limited by the type of genetic markers available (McLaren, '76; Gearhart and Oster-Granite, '78). Few markers can distinguish cells of the two genotypes *in situ* in sectioned material, and none of these has proved to be applicable to more than a limited selection of cell types (Condamine et al., '71; Dewey et al., '76; Oster-Granite and Gearhart, '81; Ponder et al., '83). It was hoped that interspecific chimeras between *M. musculus* and *M. caroli* would provide this long-sought ubiquitous marker system. In birds, an in situ marker system based on differences in nuclear morphology has been widely used in analysis of cell lineage development in interspecific chimeras between chick and quail (Le Douarin, '80). Although *M. musculus* and *M. caroli* have very similar karyotypes, their satellite DNA sequences are known to have diverged considerably (Sutton and McCallum, '72; Rice and Straus, '73). Further understanding of the evolutionary mechanisms behind this divergence is of interest because of the light it may shed on the role of centromeric DNA differences in speciation. However, the existence of such differences also suggested that it might be possible to develop an interspecific nuclear marker system in this mammalian system.

Attempts at producing interspecific hybrids between *M. musculus* and *M. caroli* were not altogether successful (West et al., '77, '78), but viable interspecific chimeras were produced (Rossant and Frels, '80). Careful analysis of the outcome of various types of pregnancy between the two species has indicated that species-specific interactions between the trophoblast and the uterus play an important role in determining the failure of interspecific hybrids and the success of interspecific chimeras.

IMPORTANCE OF TROPHOBLAST IN DETERMINING SURVIVAL OF INTERSPECIFIC HYBRIDS AND CHIMERAS

Since *M. musculus* and *M. caroli* will not naturally mate, artificial insemination of *M. musculus* females with sperm from *M. caroli* males was used to circumvent any behavioral barriers to successful reproduction between the two species (West et al., '77, '78). Only four live hybrid offspring were produced out of a very large number of attempts. It was clear from investigation of preimplantation development that many hybrid embryos were retarded when compared with embryos of either parental strain (Frels et al., '80), suggesting that there might be intrinsic problems of genomic compatibility at sperm-egg fusion or shortly thereafter. However, some embryos appeared perfectly normal at the blastocyst stage and were capable of postimplantation development.

Few such embryos proceeded through to term; the majority were resorbed between days 9 and 15 of pregnancy. The time of death

could not be correlated with any obvious developmental event, nor were there any gross abnormalities observed in the hybrid embryos prior to death (West et al., '78). It was thus suggested that failure of hybrid development late in pregnancy was due to extrinsic problems of genomic incompatibility between the embryo and the *M. musculus* uterus (Frels et al., '80). A similar proposal had been put forward previously to explain the failure of interspecific hybrids between sheep and goat and between mink and ferret (Chang and Hancock, '67). If such extrinsic interactions were largely determining hybrid embryo survival, one would predict that transfer of embryos of entirely the opposite species type should lead to the same problems. Indeed, this was found to be the case; *M. caroli* blastocysts transferred to the *M. musculus* uterus also failed to survive to term. Most embryos implanted but died between 10.5 and 13.5 days of development (Frels et al., '80; Croy et al., '82). All further experiments designed to elucidate the nature of the extrinsic problems resulting in high fetal death in interspecific hybrids were therefore performed using the interspecific embryo transfer system instead. This enabled separation of the intrinsic and extrinsic factors involved in poor hybrid survival.

There are various ways in which failure of correct fetal-uterine interaction could result in embryo death. In several interspecific embryo transfer systems, failure of trophoblast-uterine interaction at implantation leads to arrest of development at this stage (Tarkowski, '62; Chang, '81). However, implantation and, indeed, placentation appeared normal when *M. caroli* embryos were transferred to *M. musculus* uteri. Failure of endocrine function in the placenta did not seem to be involved either, because supplementation with exogenous progesterone did not promote embryo survival (Rossant et al., '82). Detailed histological examination of transferred fetuses prior to death revealed no obvious morphological abnormalities in either the fetus or the placenta. This, combined with the variable time of onset of embryonic failure, led us to suggest that maternal immune responses against the foreign species antigens might be involved in embryo death (Frels et al., '80; Croy et al., '82). Histological evidence of a maternal antifetal immune response has been previously reported for sheep-goat pregnancies (Tucker et al., '71; McGovern, '73), but the present interspecific embryo transfer system is the first in which direct functional evidence for production of maternal cytotoxic lymphocytes has been reported. Histological evidence of lymphocyte infiltration into the trophoblast surrounding the *M. caroli* fetus was observed at 9.5 days of pregnancy, before any sign of embryonic death, and maternal T-cells cytotoxic to *M. caroli* target cells in vitro were isolated from dying fetuses from 10.5 to 12.5 days of pregnancy (Croy et al., '82). No such evidence of cytotoxic infiltration was found in successful allogeneic pregnancies, even if contained in the same uterus as the resorbing *M. caroli* fetuses. Thus, maternal cytotoxic immune responses can be generated against embryonic tissue of different species genotype. So far, however, the experiments cannot determine whether the immune response observed is the primary cause of fetal death or a secondary response to some other as yet undetermined failure of embryo-maternal interaction.

Whatever the primary cause of *M. caroli* fetal death, it is clear that failure is mediated by the trophoblast layer. This has been shown by a series of experiments involving the production of chimeras between the two species. Enhanced survival of injection chimeras over aggregation chimeras in the rat-mouse interspecific system had previously suggested a role for trophoblast in protecting foreign species cells in the uterine environment (Gardner and Johnson, '73, '75; Rossant, '76). Viable interspecific chimeras between *M. musculus* and *M. caroli* were first produced by injecting *M. caroli* inner cell masses (ICMs) into *M. musculus* blastocysts and transferring the resulting composite embryos back into *M. musculus* recipients (Rossant and Frels, '80). This experiment showed that *M. caroli* ICM-derived cells could survive in the *M. musculus* uterus, if protected by *M. musculus* cells. Protection was not mediated by the presence of *M. musculus* ICM cells, because the reverse injection chimera, in which *M. musculus* ICM cells were injected into *M. caroli* blastocysts, failed to survive to term (Rossant et al., '82; Fig. 1). Final proof that trophoblast alone mediates survival or death of *M. caroli* ICM-derived cells, required production of reconstituted blastocysts in which the trophoblast was entirely of *M. musculus* genotype and the ICM was entirely *M. caroli*. We have recently performed a series of such experiments (Table 1), and have shown that viable embryos, which were entirely *M. caroli* by glucose-phosphate isomerase and phosphoglycerate kinase phenotype, could be found at

MODELS OF MUS CAROLI → MUS MUSCULUS PREGNANCY

Methods Used	Blastocyst Composition	Post-Implantation Trophoblast	Post-Implantation Embryo	Outcome
A) INSEMINATION		CM	CM	4/404 survive Most die 11 - 14 days
B) BLASTOCYST TRANSFER		C	C	1/396 survive Most die 10 - 15 days
C) C → M BLASTOCYST INJECTION		M	C+M	Viable offspring
D) M → C BLASTOCYST INJECTION		C	C+M	Death 14 - 16 days
E) M ↔ C AGGREGATION CHIMERA		C+M	C+M	Viable offspring
F) TROPHOBLAST VESICLE RECONSTITUTION		M	C	Viable offspring

Fig. 1. Models of *Mus caroli* ↔ *Mus musculus* pregnancy.

TABLE 1. Success of development of blastocysts reconstituted with M. musculus *trophectoderm and* M. caroli *ICM*

Experiment	No. transferred	No. implanted	No. live *M. caroli* embryos 5.5 d	7.5 d	9.5 d	11.5 d	15.5 d	Term
1	4	3		3				
2	9	4		1				
3	5	4				2		
4	5	4					1	
5	10	7	4					
6	10	7		4				1
Total	43	29	4	7	1	2	1	1

all stages of pregnancy. One live *M. caroli* offspring was delivered by cesarian section at 18 days of pregnancy, showing that complete protection of the *M. caroli* fetus could be provided by *M. musculus* trophoblast. The rate of successful development in these experiments was not high (Table 1), but is consistent with the rate of success reported from intraspecific reconstituted blastocysts (Gardner et al., '73; Papaioannou, '82). We predict that improved survival of interspecific hybrids could be produced by injecting hybrid ICMs into *M. musculus* trophoblast vesicles, but we have not yet attempted this experiment.

Viable interspecific chimeras were also produced after aggregation of eight-cell embryos of the two species. In this case, both trophoblast and ICM are expected to be mosaic, suggesting that it is not necessary for the entire trophoblast layer to be *M. musculus* for survival of *M. caroli* ICM-derived cells in the *M. musculus* uterus. However, al-

though we have shown that the ectoplacental cone at 9.5 days is mosaic in a majority of aggregation chimeras (Rossant et al., '82), we have not yet been able to prove that those animals that survive to term actually contain *M. caroli* trophoblast cells in the placenta. It is clearly important to establish whether the presence of some *M. caroli* trophoblast cells is compatible with chimera survival, but currently available isozymal markers do not allow one to identify the genotype of trophoblast in the mixed tissue of the term placenta.

PROPERTIES OF ISOLATED *M. CAROLI* AND *M. MUSCULUS* TROPHOBLAST

The previous experiments have emphasized the importance of correct species-specific interaction between trophoblast and uterus for survival of foreign species embryos (Fig. 1). We have compared various properties of isolated trophoblast from *M. musculus* and *M. caroli*, particularly in relation to their interaction with *M. musculus* immune system, in order to gain an insight into which aspects of trophoblast-uterine interactions are disturbed in these interspecific transfers. We have shown that *M. caroli* trophoblast is equally as active as *M. musculus* trophoblast in directly suppressing the generation of *M. musculus* cytotoxic cells in vitro (Croy et al., '83). In vivo, however, *M. caroli* embryos do not recruit local uterine suppressor cells in the same manner as *M. musculus* embryos (Clark et al., '83). Fewer cells are associated with the decidua of morphologically normal *M. caroli* embryos at 9.5 days of pregnancy, and these cells show less suppressive activity per cell than equivalent cells isolated from the decidua of *M. musculus* embryos contained in the same uterus. It has been proposed that these intrauterine suppressor cells are involved in immune protection of the antigenically dissimilar fetus in normal allogeneic pregnancies (Slapsys and Clark, '82), and their absence from *M. caroli* decidua prior to embryo death suggests that this may be one of the factors allowing generation of cytotoxic cells against the *M. caroli* fetuses.

We have also examined the fate of isolated 6.5-day *M. caroli* ectoplacental cones (EPCs) in ectopic grafts to the kidney of *M. musculus* hosts, following a report that, although allogeneic trophoblast is not rejected in the kidney of immune mice, mouse trophoblast does stimulate a graft rejection response in the rat (Simmons and Russell, '67). Histological study of grafts of *M. caroli* EPC to the kidney of either virgin or preimmunized recipients revealed no obvious difference between the fate of these grafts and similar grafts of allogeneic *M. musculus* EPC. In nonimmunized hosts, there was no sign of infiltration by lymphocytes before the ninth day after grafting, but in preimmunized hosts, extensive white blood cell accumulation was observed at the graft site from as early as 5 days after grafting. This was true for both allogeneic and xenogeneic grafts, although such evidence of a host response has not been previously reported in allogeneic grafts (Simmons and Russell, '66). In both cases, however, apparently viable giant trophoblast cells were observed, suggesting that such cells are resistant to attack by the host's graft rejection response. It is very difficult, however, to score whether trophoblast cells can ever be actually rejected, since they have an intrinsic life-span of 11–14 days even in syngeneic grafts.

Although there was no morphological difference between the host response to grafts of *M. caroli* or *M. musculus* trophoblast, the cells that invaded the graft site were shown to differ. No cells with cytotoxic properties were isolated from grafts of allogeneic or xenogeneic embryonic or trophoblast tissues to nonimmunized hosts, nor from allogeneic trophoblast in immunized recipients, but cells cytotoxic to *M. caroli* target cells were isolated from grafts of *M. caroli* EPC to preimmunized hosts (Table 2; Croy et al., in preparation). This indicates that there is a surface antigenic difference between *M. caroli* and *M. musculus* trophoblast that can be recognized by the *M. musculus* immune system. Since this recognition involves generation of cytotoxic cells, it is tempting to suggest that a similar type of response might be involved in causing the death of *M. caroli* fetuses in the *M. musculus* uterus. However, several facts about the system argue against this conclusion. First, the cytotoxic response observed against ectopic grafts of *M. caroli* trophoblast was not observed in nonimmunized hosts, and yet *M. caroli* embryos die in *M. musculus* recipients tht have never previously seen *M. caroli* antigens. Second, in the uterus, both diploid and giant trophoblast cells are potentially exposed to the maternal immune system, whereas the response observed in ectopic grafts is only to one type of trophoblast cell, the giant cell. A difference in the response to trophoblast in utero and in ectopic sites is shown by the different specificity of the cytotoxic cells isolated from

dying *M. caroli* embryos in the uterus and from *M. caroli* trophoblast grafts in the kidney (Table 3). The cells isolated from the kidney are specific for *M. caroli* targets, but the cells isolated from the uterus show a wide range of cytoxicity similar to the pan-cytotoxic T-cell described by Mason and coworkers (Dallman and Mason, '82). The kidney graft experiments do, however, show that there are surface specificities on *M. caroli* trophoblast that are different from those of the *M. musculus* species. Although it is still unclear whether these differences directly result in immune destruction of the *M. caroli* embryo in the *M. musculus* uterus, they may represent important cell-surface molecules normally involved in promoting correct trophoblast-uterine interaction. Major differences in such molecules between the two species may lead to breakdown of the normal trophoblast-uterine interface and promote exposure of *M. caroli* antigens which can be recognized and destroyed by the *M. musculus* immune system. We shall attempt to characterize further the cell-surface differences between *M. caroli* and *M. musculus* trophoblast by raising monoclonal antibodies against EPC tissue from the two species.

PREGNANCIES IN INTERSPECIFIC CHIMERAS

Female interspecific chimeras provide a potentially useful system for assessing the relative importance of maternal immune rejection and incorrect trophoblast-uterine

TABLE 2. Cytotoxic activity in cells isolated from xenografts of Mus caroli *embryonic and ectoplacental cone tissues to kidney*[1]

Experiment	Recipient	Graft	Cytotoxicity[2] against *M. caroli* blasts
1	Nonimmune	EPC	<SR[3]
		E	0
	Preimmune[4]	EPC	+++
		E	+
2	Preimmune[5]	EPC	+++
		E	<SR

[1]6.5-day *M. caroli* conceptuses were dissected into ectoplacental cone (EPC), embryonic region (E), and extraembryonic region. E or EPC was grafted beneath the kidney capsule of Ha (ICR) mice, and 5 days later cells from the graft sites were tested in a ^{51}Cr release assay.
[2]3×10^3 *M. caroli* Con A lymphoblasts were incubated in "V" bottom microtest plates for 6 h. In Exp. 1 effector/target ratio was 20:1, and in Exp. 2 effector/target ratio was 15:1.
[3]Percent specific ^{51}Cr released. <SR = less than spontaneous release in the presence of cells from graft sites of Ha (ICR) embryos; + = 5–10% release, +++ = release of 20% or more.
[4]Graft recipients had received *M. caroli* lymphocytes as neonates and subsequently rejected *M. caroli* skin when tested at 6 weeks of age.
[5]Graft recipients had received three injections of *M. caroli* lymphoid cells.

TABLE 3. Specificity of cytotoxic cells induced by Mus caroli

^{51}Cr-labeled[1] target	CTL generated[2] in vitro (CD1 α caroli)	Cells isolated[3] from grafts to kidneys of preimmune mice		Cells isolated[3] from *M. caroli* resorbing in utero
		E	EPC	
M. caroli blast	+++[4]	0	++	+
P815 mastocytoma	++	0	0	+
DBA/2J blast	+/−	0	0	+
CD1 blast	0	0	0	n.t.[5]
C3H/He blast	n.t.	n.t.	n.t.	+
YAC	++	0	0	+

[1]3×10^3 target cells were cultured for 6 h in "V" bottom microtest plates with effector cells. Effector cell-to-target cell ratios were 15:1 or 20:1. Lymphoblasts were induced using concanavalin A (Difco Laboratories).
[2]Tube cultures containing 3×10^6 CD1 spleen cells and 10^6 irradiated *M. caroli* spleen cells in 3 ml of α-MEM + 10% FCS, 20 mM HEPES, and 10^{-5} M 2-mercaptoethanol.
[3]Cell suspensions prepared by preliminary separation on Lympholyte M (Cedarlane Laboratories).
[4]Percent specific ^{51}Cr released: +/− = <5%; + = 5–10%, ++ = 10–20%, +++ = >20%.
[5]Not tested.

interaction in the failure of *M. caroli* fetuses. If immune rejection is the primary cause of death, both *M. caroli* and *M. musculus* embryos should survive in interspecific chimeras, because chimeras are expected to be immunologically tolerant to the two parent species (Matsunaga et al., '80). On the other hand, if correct species-specific trophoblast-uterine interaction is required for embryo survival, *M. caroli* embryos will only survive in uteruses of chimeras which contain patches of *M. caroli* uterine epithelium. When interspecific chimeras were naturally mated with *M. musculus* males, interspecific hybrids were found among their offspring in greater numbers than ever produced by artificial insemination (Rossant and Chapman, '83), supporting the immunological hypothesis. However, preliminary studies on transfer of *M. musculus* and *M. caroli* blastocysts to interspecific chimeras have produced less clear-cut results. To date, three chimeras have given birth after transfer of *M. musculus* and *M. caroli* embryos to opposite uterine horns, and all three have produced the full complement of *M. musculus* offspring and no *M. caroli* offspring. Laparotomy during gestation revealed that the *M. caroli* embryos were resorbing around day 13 of pregnancy. This result is difficult to explain on either hypothesis. Unless interspecific chimeras are not truly immunologically tolerant to both species, immune reaction against the *M. caroli* embryos cannot be causing their death. We are currently assessing the immunological status of the chimeras to determine if the immune tolerance observed in intraspecific chimeras extends to chimeras between species. Preliminary evidence from skin grafting and mixed lymphocyte responses indicates that there may be some recognition of both parental species in the chimeras (Croy and Rossant, unpublished). However, it is not yet clear that this recognition extends to the generation of the type of cytotoxic cells expected to be involved in rejection of the fetus. Incorrect trophoblast-uterine interaction leading to fetal death also does not correctly predict the pattern of fetal death observed in chimeras. The chimeras used for transfer were balanced in their contributions from the two species; one would expect the uterus to contain patches of cells of either species so that at least some *M. caroli* embryos could find a suitable site for implantation and development. Conversely, one would predict that some *M. musculus* embryos would attach to *M. caroli* uterine epithelium and fail to develop. This latter prediction depends on the assumption that embryo failure is reciprocal in this interspecies pregnancy system, which, for technical reasons, has not yet been tested. It is important to examine the actual genotype of the uterine cells in contact with *M. caroli* conceptuses in the chimeras. This study is being undertaken using the cell marker system described in the following section.

Thus, although the *M. musculus–M. caroli* pregnancy system has clearly pinpointed the importance of the trophoblast-uterine interaction in promoting embryo survival, the exact cause of death of *M. caroli* embryos in the *M. musculus* uterus is still unclear. Maternal immune responses are involved in fetal death but may not be the only cause of death. Further study of this system is required to elucidate which aspects of trophoblast-uterine interaction fail. Understanding this problem will be important not only for the light it may shed on the mechanisms involved in normally preventing interspecific pregnancies, but also for the insight it may provide into the role of the trophoblast in protecting the fetus in pregnancies within species.

INTERSPECIFIC CHIMERAS AS TOOLS IN EMBRYOLOGY

Despite the problems involved in interaction between trophoblast of *M. caroli* and uterus of *M. musculus*, it is clear that the ICM-derived cells of the two species can interact together perfectly normally in chimeras. It appears that mammals can tolerate a greater degree of genetic disparity between the uterus and the ICM than between the uterus and the trophoblast. This does not mean, however, that ICM cells of any species will survive in the *M. musculus* uterus if surrounded by *M. musculus* trophoblast. The species must probably be quite closely related. For example, rat ICM cells will survive to midgestation in the mouse blastocyst but are selected out as development proceeds in the mouse uterus (Gardner and Johnson, '75). No such selection can be detected against *M. caroli* cells in interspecific chimeras (Rossant and Frels, '80), and extensive analysis of mosaicism has shown that *M. caroli–M. musculus* chimeras resemble *M. musculus–M. musculus* chimeras in every respect (Rossant and Chapman, '83). The interspecific chimeras between *M. musculus* and *M. caroli* thus provide a useful model system to study the development of cell lineages during development, because the genetic disparity between the two species allows the identifi-

cation of cells in situ in sections of chimeric material, but the genetic relatedness allows extrapolation of the results observed to normal embryonic development.

We have recently exploited differences in satellite DNA sequences between *M. musculus* and *M. caroli* to generate a ubiquitous in situ marker system (Siracusa et al., '83; Rossant et al., '83). A cloned probe to *M. musculus* satellite DNA was labeled with ^{3}H-thymidine and hybridized in situ to sections of chimeric material. Autoradiography allowed clear distinction between the labeled *M. musculus* cells and unlabeled *M. caroli* cells. We have already used the marker to determine directly for the first time the derivatives of the ICM and the trophectoderm in the early postimplantation embryo (Rossant et al., '83).

This marker system has several advantages over other in situ marker systems, the chief of which is its ubiquity. All cells that contain a nucleus should be amenable to analysis. A similar approach has been used to distinguish rat and mouse cells in chimeric combinations (Müller et al., '82), but previous evidence for cell selection acting in the rat-mouse embryonic chimera system (Gardner and Johnson, '73, '75) makes the rat-mouse marker unsuitable for study of embryonic cell lineages in vivo. We hope that the marker system described for *M. caroli–M. musculus* chimeras will be of wide use to workers interested in various aspects of cell lineage development.

IN SITU MARKER ANALYSIS OF CHIMERAS: INFORMATION RELEVANT TO THE MECHANISM OF PROTECTION BY THE TROPHOBLAST

We have begun to use the in situ marker system to investigate more closely the relationship between the genotype of the trophoblast and the survival of *M. caroli* ICM-derived cells in the *M. musculus* uterus. The production of a viable *M. caroli* fetus from a reconstituted blastocyst was the definitive proof of the overriding importance of trophoblast genotype in promoting fetal survival. This implies that the portion of the mature chorioallantoic placenta that is derived from the ICM does not affect fetal survival. Descriptive studies do not allow one to determine what proportion of the fetal side of the placenta consists of the ICM-derived tissue, but the success of the reconstituted blastocyst experiments suggest it may not be a very significant proportion. This has been confirmed by in situ analysis of the placenta obtained from the surviving *M. caroli* fetus. Very few unlabeled *M. caroli* ICM-derived cells were detected in the bulk of the chorioallantoic placenta, except for cells of the fetal blood vessels and the parietal yolk sac, which impinges on the placenta at the base of the umbilical cord.

This study has also made it possible to determine the genotype of the trophoblast layer in surviving aggregation chimeras, where isozyme analysis cannot reveal whether all the trophoblast is of *M. musculus* type. Since we now know which parts of the placenta are ICM-derived, we should be able to distinguish any *M. caroli* contribution to the trophoblast in aggregation chimeras. We have not yet looked at the placenta of chimeras at term, but preliminary analysis of the placenta at 9.5 days confirms that both *M. caroli* and *M. musculus* cells can contribute to the trophoblast. However, in the chimera examined in most detail so far, *M. caroli* cells were only found in the diploid core of the ectoplacental cone and not in the giant cells in direct contact with the maternal environment (Fig. 2). Further analysis is clearly required, but it does suggest that there may be selection against the presence of *M. caroli* trophoblast cells at the fetal-maternal interface in aggregation chimeras.

The distribution of *M. musculus* and *M. caroli* cells in the embryonic tissues of the aggregation chimeras also reveals some interesting information. Short-term studies on aggregation chimeras have revealed that little cell mixing occurs during the early stages of development (Garner and McLaren, '74), although adult chimeras show fine-grained mosaicism. It is not yet clear when cell mixing occurs during development (McLaren, '76; West, '78). Little cell mixing was apparent in the ICM derivatives of *M. caroli–M. musculus* injection chimeras at 7 days of development (Rossant et al., '83, and unpublished observations). However, the embryonic tissues of the 9.5-day aggregation chimeras examined showed evidence of extensive cell mixing throughout. Within the nervous system, there was some evidence for a renewal of clonal growth, since fairly large patches of cells of one genotype could be observed (Fig. 3), but elsewhere, cells of the two genotypes were apparently randomly distributed. The time and extent of cell mixing has considerable implication for understanding establishment of cell lineages, so further analysis of intermediate stages between day 7 and day 9 will be undertaken to discover when this occurs.

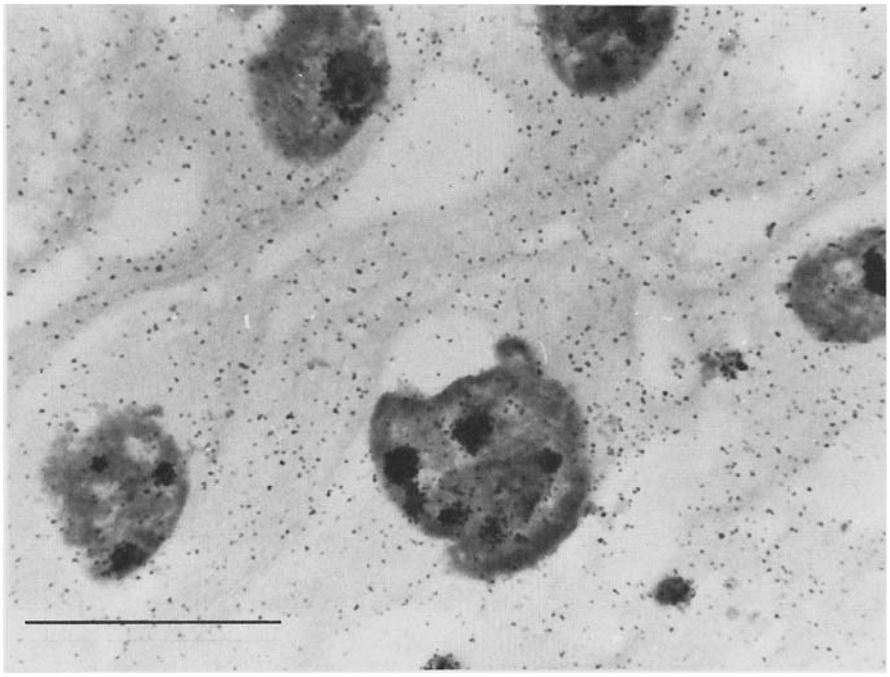

Fig. 2. In situ hybridization of *M. musculus* satellite DNA probe to giant trophoblast cells of *M. musculus* ↔ *M. caroli* aggregation chimera at 9.5 days of pregnancy. All giant cells in this and other sections were labeled, although unlabeled cells were observed in the diploid portion of the ectoplacental cone. Note close association of satellite DNA hybridization with heterochromatin of giant nuclei. Grid bar = 50 μm.

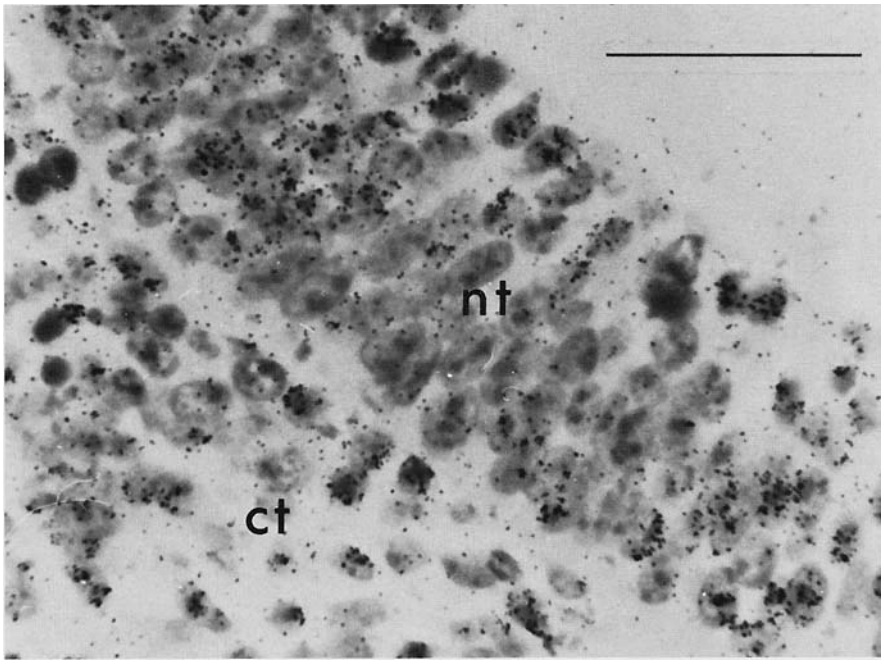

Fig. 3. In situ hybridization of *M. musculus* satellite DNA to embryonic tissues of same chimera. Note apparent clonal growth in neural tube (nt) as indicated by confluent groups of labeled and unlabeled cells and more random intermixing of cells in adjacent connective tissue (ct). Grid bar = 50 μm.

CONCLUSIONS

Interspecific pregnancies between *M. caroli* and *M. musculus* have provided useful tools for studying various aspects of mammalian development. Production of viable interspecific hybrids has been enhanced by breeding interspecific chimera females, and examination of a variety of pregnancies between the two species has cast some light on the possible extrinsic problems that may be encountered by mammalian hybrid embryos during uterine development. All hybrids produced to date have been sterile, so the original aim of introducing new genetic polymorphisms into the *M. musculus* gene pool has not been realized. However, the hybrid animals themselves are proving very useful genetic tools. Interspecific chimeras between *M. musculus* and *M. caroli* are equally useful tools for the study of embryology, because of their similar development to intraspecific chimeras and the ability to distinguish cells of the two species in situ. These interspecific pregnancies have thus provided us with tools for addressing problems in genetics, embryology, and reproductive immunobiology that could not readily be addressed in intraspecific pregnancies.

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