Immune presensitization and local intrauterine defences as determinants of success or failure of murine interspecies pregnancies

D. A. Clark*, B. A. Croy†, J. Rossant‡ and G. Chaouat‡

*Department of Medicine, McMaster University, 1200 Main Street West, Hamilton, Ontario, Canada L8N 3Z5; †Department of Biological Sciences, Brock University, St Catharines, Ontario, Canada L2S 3A1; and ‡Institut Curie, 26 rue d'Ulm, 75231 Paris Cedex 05, France

Summary. Putatively immuno-incompetent *Mus musculus* females exhibited failure to support pregnancy of *Mus caroli* embryos. These results for *M. musculus* females (i.e. treated by cyclosporine A, of the *nu/nu* genotype, and as an interspecies chimaera) can be explained in immunological terms. *Mus musculus* females possessed pre-sensitized cytotoxic T cells against *Mus caroli* antigens. *Nu/nu* mice possessed activated NK cells and macrophages, and selectively discriminated against *Mus caroli* embryos early in pregnancy unlike normal *+/+* females; the requirement for T cells to activate non-specific cytotoxic effector mechanisms was bypassed in *nu/nu* mice. *Mus caroli* are not inbred, and interspecies chimaeras which are tolerant of the antigens on the *Mus musculus* donor strain were not tolerant of cells from unrelated *Mus caroli*. Interspecies chimaeras also behaved as if they were pre-sensitized to *Mus caroli*. Our results show that *Mus caroli* embryos recruit fewer active suppressor cells even when gestating in *Mus caroli* decidua as compared to *Mus musculus* embryos in *Mus musculus* decidua and that the ability of *Mus caroli* placental cells to directly inhibit cytotoxic effector cell killing was inherently less than the inhibitory activity of placental cells from *Mus musculus*. *Mus caroli* embryos therefore appear to be less well defended against maternal immune attack even when gestating in a uterus possessing compatible *Mus caroli* decidual tissue.

Introduction

Semiallogeneic pregnancy which occurs as a result of mating between histoincompatible individuals belonging to the same species is generally successful and there are few animal models with which natural mating leads to a moderate abortion rate (Clark, 1985; Clark *et al.*, 1985). Many types of interspecies pregnancies, in contrast, tend to have a high failure rate. Sheep × goat matings, and pregnancies created by transferring donkey embryos into horses, usually result in intrauterine destruction of the embryo associated with an infiltration by maternal mononuclear cells at the endometrial–trophoblastic interface reminiscent of the histological picture of graft rejection (Hancock, McGovern & Stamp, 1968; Allen, 1982). Furthermore, repeat pregnancies of the same type fail more rapidly and a similar accelerated abortion can be achieved by immunization with the cells of the male, a finding suggestive of immunological memory (McGovern, 1973; Allen, 1982). However, some types of interspecies blastocyst transfers do lead to successful pregnancy (Allen, 1982; Bennett & Foster, 1985; Kydd, Boyle, Allen, Shepherd & Summers, 1985) and it is important to understand the mechanisms that determine the success of xenoembryo transfer as a prelude to preserving endangered species by blastocyst transfer to xenogeneic recipients.

We have investigated a murine model of xenogeneic pregnancy failure created by transferring

blastocysts from one species of mouse (*Mus caroli*) into the uterus of another species, the laboratory mouse (*Mus musculus*). The *Mus caroli* embryos implant and develop for about 5 days whereupon they are infiltrated by maternal mononuclear cells and resorb. Although the nature of the initial infiltrate is unclear, at later times when the embryos are necrotic we have demonstrated that maternal cytotoxic T lymphoblasts (CTL) with a degenerate (Wilson & Shortman, 1984) or pan-specific pattern of killing are present within the dead embryos (Croy, Rossant & Clark, 1982, 1985; Clark, Slapsys, Croy & Rossant, 1984).

Mus caroli embryos will survive to term in the *Mus musculus* uterus if enveloped in *Mus musculus* trophoblast. Therefore, the trophoblast must play a key role in determining the outcome of the xenopregnancy (Rossant, Croy, Clark & Chapman, 1983). During successful semiallogeneic pregnancy, non-T suppressor cells develop in uterine decidua at the implantation site. Local active suppressor cells are markedly scarce at the *Mus caroli* implant sites in the *Mus musculus* uterus 5 days after implantation (Clark *et al.*, 1983). Since these local suppressor cells appear to be activated or recruited by trophoblast cells, we have proposed that the *Mus caroli* embryos may be immunologically rejected due to a species restriction that prevents suppressor cell recruitment by the xenogeneic trophoblast cells (Clark *et al.*, 1983; Clark & Slapsys, 1985; Slapsys, Beeson & Clark, 1985). However, failure of trophoblast for biological reasons in the xenogeneic environment could result in embryo death due to malnutrition and anoxia, with the trophoblast becoming permeable to infiltrating leucocytes and lymphoid cells. Embryonic death might then occur whether or not there was a maternal immune response (Clark, 1985).

In the present study we have investigated the immunological basis for the failure of *M. caroli* embryos in putatively immunoincompetent *M. musculus* mice.

Materials and Methods

Mice. Embryo transfer and chimaera production using outbred *Mus caroli* and *Mus musculus* have been described in detail elsewhere (Croy *et al.*, 1982, 1985). CDI and CDI *nu/nu* mice were purchased from Charles River; DBA/2 and C3H/He mice were obtained from the Jackson Laboratories, Bar Harbor, Maine. C3H $\times$ DBA/2 (C3D2F₁) mice were bred in our unit. C3H/He, BALB/c, and *Mus caroli* were also obtained from the IRCS breeding facility at Villejuif and CSEAL-CNRS (Orleans) breeding unit from the nucleus of Dr J. Bonhomme.

Immunological methods. Delayed-type hypersensitivity was tested by the foot-pad swelling technique. Briefly, the thickness of the right and left hind footpads was measured using a pressure sensitive micrometer, and 20 μ l of medium or test cells (usually spleen cells) were injected into the right and left pad. After 24 h, the thickness was again measured and the difference in swelling due to the cellular challenge (i.e. increment with cells – increment with medium alone) was determined. Medium used throughout the study was alpha-MEM supplemented to contain 10% fetal bovine serum, 100 i.u. penicillin/ml, 100 μ g streptomycin/ml, and 5×10^{-5} M-2-mercaptoethanol. Sterile cell suspensions were prepared as previously described by gently pressing the spleens through a 60-mesh stainless-steel screen (Clark *et al.*, 1983).

Mixed lymphocyte cultures were performed by mixing 3×10^6 spleen cells from one type of mouse (the responder) with 1×10^6 irradiated histoincompatible spleen cells (stimulator cells) in 3 ml medium in 17 $\times$ 100 mm plastic tubes. After 4–5 days at 37°C in 7% CO₂, the cell pellet was tested for its ability to lyse ⁵¹Cr-labelled tumour cells (YAC) or lymphoblasts that had been generated by growing spleen cells from the stimulator strain of mouse in medium with concanavalin A for 3 days as described elsewhere (Croy *et al.*, 1982). After 4 h incubation at 37°C, an aliquant of supernatant was removed and the c.p.m. of ⁵¹Cr were measured in a gamma counter. Percentage specific release was determined using the standard formula,

$$P = 100\% \times \frac{(\text{c.p.m. released with MLC cells} - \text{c.p.m. released spontaneously})}{(\text{c.p.m. released by NP-40} - \text{c.p.m. released spontaneously})}$$

In some experiments the titration curves were computer analysed to determine cytotoxic cell activity in terms of lytic units (Clark *et al.*, 1983).

A soluble suppressor factor was prepared from cell suspensions made from the decidua of Day 14.5–15.5 allopregnant *Mus musculus* or *Mus caroli* (i.e. *Mus caroli* × *Mus caroli*) (Slapsys & Clark, 1983; Clark, Chaput, Walker & Rosenthal, 1986). The cell-free supernatants were tested for their ability to suppress the generation of cytotoxic cells in the mixed lymphocyte cultures.

Limiting dilution experiments were conducted to determine the frequency of CTL precursors against *Mus caroli* and against *Mus musculus*. Small numbers of responding spleen cells were incubated in V-bottom microtray wells with 2×10^5 irradiated stimulator cells plus a 10% concentration of lectin-free interleukin 2 (IL-2) (Cellular Products, Buffalo, NY, U.S.A.). IL-2 provides the 'help' needed for the precursors of CTL to proliferate into a clone of sufficient size to be detectable. Detection of CTL activity was achieved by adding 2000 ^{51}Cr -labelled target cells to each well after 6 days of culture and those wells showing ^{51}Cr -release greater than the spontaneous release + 2 standard deviations after 4 h at 37°C were scored as positive. The frequency of precursors was calculated by fitting the data to the Poisson formula by using the method of maximum likelihood (Porter & Berry, 1964).

In some experiments, a C3H embryonic cell line with trophoblastic features derived from placenta ('B6' cells) (Beeson, Daynes, Weinert & Gahring, 1984) were used as target cells to test for effector activity. This was done either by ^{51}Cr -release as described above or by measuring the effect of irradiated (1500 R ^{60}Co) effector populations on proliferation of B6 cells in U-bottomed 96-well trays as assessed by incorporation of [^3H]thymidine. Briefly, 1×10^4 freshly trypsinized cultured 'B6' cells were seeded with or without splenocytes and after 48 h 0.1 μCi [^3H]thymidine was added. After 12–18 h, the incorporated counts were determined by harvesting on to a glass fibre filter (Skatron), and counting in a liquid scintillation counter.

Placental cell suspensions from Day 14.5–15.5 of pregnancy were presented by collagenase digestion as described elsewhere (Chaouat & Kolb, 1984) and tested for their ability to block lysis of YAC target cells by lymphokine-activated killer cells (LAKs). The LAKs were generated by incubating spleen cells for 4–7 days in culture medium supplemented with IL-2 derived from PMA-stimulated EL-4 cells (Kolb, Chaouat & Chassoux, 1984). This IL-2 was kindly provided by Dr J. P. Kolb, Institut Curie, Paris, France. The washed LAKs were added with placental cells to the assay wells containing ^{51}Cr -labelled YAC targets, and 4 h later, the amount of isotope release was determined.

Results

Mus musculus are presensitized to *Mus caroli*

It has previously been reported that *Mus caroli* xenoembryos invariably failed in cyclosporine A-treated *Mus musculus* females, no matter how high the dose of drug (Croy *et al.*, 1985). However, once the dose had reached 15 mg/kg/day, normal alloembryos began to die and at a dose of 50 mg/kg/day, there was complete lethality in agreement with the observations of Ryffel (1982). It was important to know whether or not the cyclosporine A was an effective suppressant of a putative *Mus musculus* anti-*caroli* immune response. Cyclosporine A at a dose of 7.5–15 mg/kg/day can effectively inhibit a primary immune response to an allograft, but is frequently ineffective if there is pre-sensitization (Homan, Fabre, Williams, Millard & Morris, 1980; Horsburgh, Wood & Brent, 1980; Gratwohl, Forster & Speck, 1981). Therefore, the foot-pad swelling test was done to test for pre-existing sensitization of *Mus musculus* against *Mus caroli* antigens. As illustrated in Table 1, in

all 3 experiments, delayed-type hypersensitivity to *Mus caroli* cells was demonstrated. In contrast, in 2 reciprocal experiments in which *Mus caroli* were tested for sensitization to *Mus musculus*, no significant hypersensitivity was found (net specific swelling of 5.0 ± 8.1 (N = 5) and 15.5 ± 10.2 (N = 5); the latter experiment contained an outlier that increased mean swelling but also the s.e.m.). These results suggested that *Mus musculus* embryos might be destroyed less rapidly in the uterus of *Mus caroli*, as previously reported (Croy *et al.*, 1985).

Table 1. Effect of challenge cells from *Mus caroli* or *Mus musculus* (CD1, C3D2F₁) on foot-pad swelling of C3H and CD1 (*M. musculus*) mice

Test animal	No. of mice	Cell challenge given	Net swelling (mean $\pm$ s.e.m.) ($\times 10^{-2}$ mm)	Net specific swelling ($\times 10^{-2}$ mm)
C3H (H-2 ^k)	4	3×10^7 <i>Mus caroli</i>	13.5 ± 3.9	$14.5 \pm 5.9^*$
	4	3×10^7 CD1 (H-2 ^b)	-1.0 ± 4.4	
C3H (H-2 ^k)	6	2×10^7 <i>Mus caroli</i>	25.0 ± 2.8	$15.2 \pm 5.6^*$
	6	2×10^7 C3D2F ₁ (H-2 ^{kd})	9.8 ± 4.9	
CD1 (H-2 ^b)	7	2×10^7 <i>Mus caroli</i>	21.9 ± 4.2	$14.5 \pm 5.4^*$
	5	2×10^7 C3D2F ₁ (H-2 ^{kd})	7.4 ± 3.4	

* $P < 0.05$ (Student's *t* test) for swelling induced by *Mus caroli* spleen compared to effect of *Mus musculus* control spleen cells.

CTL precursor frequency as a test for presensitization

Unidirectional graft rejection models have been described for thyroid and islet cell allografts in B10.S and B10.S (7R) mice in which a graft in one direction fails (B10.S into B10.S (7R)) but succeeds in the opposite direction (B10.S (7R) into B10.S) (Isakov & Bach, 1985). The unidirectional rejection was explained by the finding that the accepting (i.e. non-rejecting) recipient had only 25% as many CTL precursors reactive with B10.S (7R) antigens as the B10.S (7R) rejecting strain had to B10.S (Isakov & Bach, 1985). Therefore, the CTL precursor frequency for *Mus musculus* and *Mus caroli* against each others antigens was determined. Table 2 illustrates that *Mus caroli* had a much lower CTL precursor frequency against C3H compared to C3H against *Mus caroli*. A similar difference in CTL precursor frequency was found when *Mus caroli* were compared to a second *Mus musculus* strain, CD1. Thus differences in CTL precursor frequencies between *Mus musculus* and *Mus caroli* were comparable to those seen in the unidirectional thyroid graft-rejection system. Furthermore, if IL-2 alone was added to the spleen cells in the absence of antigenic stimulators, C3H produced cytotoxic effectors that specifically killed *Mus caroli* lymphoblasts whereas *Mus caroli* spleen cells incubated in IL-2 failed to produce any killer activity (Table 2). Similar results were obtained using CD1 + IL-2. The activation of specific killing by IL-2 has been shown to result from the reactivation of memory CTL cells (Yoshimoto, Kashima, Okada, Amikura & Hamuro, 1985).

Therefore, both the foot-pad swelling data and the CTL precursor data indicated that *Mus musculus* possessed pre-existing immunity to *Mus caroli*. This pre-existing immunity could explain the failure of cyclosporine to block resorption of *Mus caroli* embryos in the *Mus musculus* uteri, the rapidity of the rejection of *Mus caroli* embryos in *Mus musculus*, and the non-reciprocal nature of the transfer system.

Studies with putatively immunoincompetent nude mice

It has previously been shown that *Mus caroli* embryos resorb in the uterus of CD1 *nu/nu* females, whereas CD1 *Mus musculus* embryos gestate successfully (Croy *et al.*, 1985). Mice of the

Table 2. Limiting dilution analysis of *Mus caroli* and *Mus musculus* CTL precursors

Responder spleen cells	Stimulator	Target	No. of responder cells per well*				CTL precursor frequency (95% confidence interval)	CTL precursor 10 ⁶ spleen cells
			3704	1235	412	137		
<i>Mus caroli</i>	C3H + IL-2	<i>M. caroli</i>	3/12	1/12	1/12	0/11	—	—
		C3H	9/12	6/12	1/12	1/12	1/2329 (1455–3709)	429
	IL-2	<i>M. caroli</i>	1/12	0/12	0/12	1/12	—	—
		C3H	2/12	2/12	0/12	0/12	—	—
C3H	<i>M. caroli</i> + IL-2	<i>M. caroli</i>	12/12	10/12	4/12	1/12	1/819 (508–1318)	1221
		C3H	1/11	0/11	0/12	1/12	—	—
	IL-2	<i>M. caroli</i>	10/12	7/12	2/12	1/12	1/1804 (1112–2927)	554
		C3H	3/12	0/12	2/11	0/12	—	—

*The no. of wells scored per total tested at the indicated number of responder cells is shown. Spontaneous release of the Con A blast target cells was determined from wells containing irradiated stimulators + IL-2 or IL-2 in medium alone.

nu/nu genotype lack an effector T cell system and will bear skin allografts and skin xenografts, including *Mus caroli*, without signs of rejection, and it could therefore be presumed that failure of *Mus caroli* in the *nu/nu* uterus is not mediated by CTL. However, *nu/nu* mice possess activated NK cells and, more importantly, activated macrophages that render them more resistant to intracellular infections and capable of rejecting transplants of xenogeneic embryonic tumours and melanomas (Solter & Damjanov, 1979; Sharp & Colson, 1984; Rousseau-Merck *et al.*, 1985; Jacobovich, Cabrillat, Gerlier, Bailly & Dore, 1985). During the allograft rejection response in normal immunocompetent mice, sensitized T cells activate macrophages and NK cells. This requirement for sensitized T cells to produce NK and macrophage activation is bypassed in *nu/nu* mice. Since the uterus is replete with macrophages (Matthews, Adams & Searle, 1985), and since macrophages have been reported to be unusually cytolytic towards xenogeneic target cells (Cabilly & Gallily, 1981), we have reviewed the data obtained from transfers of *Mus caroli* and *Mus musculus* blastocysts to the *+/+* and *nu/nu* uterus for evidence of selection against xenogeneic *Mus caroli* embryos in *nu/nu* recipients.

The implantation rate of *Mus caroli* as compared to *Mus musculus* in *+/+* recipients in experiments done over a 1½- to 2-year period was similar (66/83 = 80% vs 70/78 = 90%). In contrast, the implantation rate as assessed on Day 10.5 in *nu/nu* recipients (Croy *et al.*, 1982) was only 61% (17/28) for *Mus caroli* blastocysts as compared to 16/19 (84%) for *Mus musculus* ($P < 0.05$ by Fisher's Exact test). A comparison of transfers over a 1½-year period for *Mus caroli* and *Mus musculus* blastocysts showed that all the *Mus caroli* embryos were dead at the time of analysis, a more rapid demise than had occurred in *+/+* recipients (Croy *et al.*, 1985). These accumulated data suggested that *nu/nu* mice differed from *+/+* animals and may have been able to discriminate against *Mus caroli* embryos at the implantation and early post-implantation stages of pregnancy.

To test for activated non-specific effector cells against embryonic placental cells in *nu/nu* mice, we used a C3H placental cell line ('B6') which grows transiently and regresses in *nu/nu* mice. B6 target cells proved susceptible to killing by LAK but not by NK cells in normal spleen (4/4 experiments) (Exp. 1, Table 3) and *nu/nu* spleen cells were cytotoxic for 'B6' targets as compared to *+/+* splenocytes (2/2 experiments) and could markedly inhibit the growth of 'B6' cells *in vitro* (3/3 experiments) (Exps 2 & 3, Table 3). The identity of the effector population in the *nu/nu* mice was not determined in these studies. The flat titration curves from cytotoxicity shown in Exp. 2 were reminiscent of the flat titration curves seen with low-level cytotoxic activity of in-vivo allosensitized cells (Clark, Phillips & Miller, 1977).

Table 3. Effect of different types of host effector cells on the ‘B6’ placental cell line *in vitro*

Test system	Effector to target ratio	% Cytotoxicity (mean ± s.e.m.) with:		
		LAK	+ / + spleen	<i>nu/nu</i> spleen
⁵¹ Cr-release, 5 h	50:1	26.9 ± 3.8	−0.9 ± 0.9	nd
	25:1	15.9 ± 1.0	0.1 ± 0.5	nd
	12.5:1	8.8 ± 1.2	0.6 ± 0.2	nd
⁵¹ Cr-release, 5 h	50:1	nd	—	10.2 ± 2.1*
	25:1	nd	—	13.2 ± 1.8
	12.5:1	nd	—	13.1 ± 1.3
³ H] Thymidine incorporation	1:1	48.8 ± 2.5	2.9 ± 6.9	47.4 ± 5.1
	1:1†	52.0 ± 2.2	13.4 ± 9.4	42.6 ± 2.6

Values are mean ± s.e.m.; nd = not done.
*c.p.m. released with +/+ spleen cells alone was used to compute spontaneous release value.
†Treated with 1500 R before addition to culture. Values represent percentage decrease in c.p.m. incorporated compared to wells containing ‘B6’ cells and medium alone.

Putatively tolerant interspecies chimaeric mice are not tolerant of Mus caroli

An alternative strategy to prevent immunological rejection of *Mus caroli* embryos is to use female recipients that are immunologically tolerant of *Mus caroli* antigens. It has been possible to create mice that are chimaeric, being composed of both *Mus musculus* and *Mus caroli* tissues. These chimaeras should be tolerant of the species antigens of both *Mus musculus* and *Mus caroli*. However, *Mus musculus* embryos were found to survive in the uterus of the chimaera whereas the *Mus caroli* embryos failed (Croy *et al.*, 1985). Before concluding that non-immunological mechanisms led to the demise of the *Mus caroli* embryos, it is important to know whether the chimaeras were, in fact, immunologically tolerant. To examine this issue, the chimaeras were tested for their ability to reject skin grafts *in vivo* and their spleen cells were tested for the ability to generate CTL *in vitro*. As shown in Table 4, the chimaeric spleen cells were able to mount a CTL response against pooled *Mus caroli* stimulator cells. Six chimaeras were tested with similar results. Furthermore, *Mus caroli* skin grafts were rejected by the chimaeras (Croy *et al.*, 1985). *Mus caroli* are not inbred and therefore these results could be explained by the skin graft donors being allogeneic to the parental contribution to the chimaera. However, addition of IL-2 to the chimaeric spleen cells also led to the generation of CTL specific for *Mus caroli* antigens (Table 4) and the splenic lymphocytes from the chimaeras therefore behaved as if they had been presensitized to *Mus caroli* lymphoblast antigens.

Mus caroli have deficient defences against rejection

Allogeneic matings are generally successful. The antigenic differences between individual *Mus caroli* animals that allow one *Mus caroli* to generate CTL against another *Mus caroli* are equivalent to allogeneic differences between different strains of inbred *Mus musculus*. Furthermore, it has been shown that CTL against xenoantigens also manifest the specificity typical of CTL against alloantigens (Burakoff, Ratnofsky & Benacerraf, 1977). CTL generated by chimaeras against either *Mus musculus* or *Mus caroli* antigens should therefore be viewed as allospecific CTL. During normal allogeneic pregnancy in *Mus musculus*, local active suppression by suppressor cells in decidua and by placental cells inhibits CTL development and killing activity (Kolb *et al.*, 1984; Clark, 1985) and in a chimaera, both *Mus musculus* and *Mus caroli* trophoblast should be able to recruit suppressors provided the decidua is chimaeric (Clark & Slapsys, 1985). This suppression in allopregnant *Mus musculus* is sufficient to prevent rejection even when the female has been immunized against the MHC antigens of the father (Clark, 1985; Clark *et al.*, 1985). Since it has been shown in Table 4

Table 4. Immune reactivity of CD1 *Mus musculus*–*Mus caroli* chimaeras

Test system	Antigen used	Target cell		
		<i>Mus caroli</i>	CD1	DBA/2
Tube MLC	CD1	4.9 ± 1%*	6.1 ± 1%	0%
	DBA/2	23.9 ± 2%	15.8 ± 1%	54.6 ± 1%
	<i>Mus caroli</i>	44.8 ± 2%	9.9 ± 0.1%	0%
Limiting	IL-2 only	1–5381 (2855–10141)*	0	0
Dilution	IL-2 only	1–6753 (3352–13601)*	0	0

Values are % specific release of ^{51}Cr from Con A lymphoblast targets (mean ± s.e.m.) at an effector to target ratio of 30:1 from one of 6 experiments testing spleen cells of individual chimaeras. Spontaneous release was determined in the presence of an equivalent ratio of cells from a syngeneic (i.e. CD1 + irradiated CD1) MLC. Of the 6 chimaeric females tested, the spleen cells of 3 were tested for composition by GPI typing. On this basis, the chimaeras were 15, 30, and 35% *caroli*. The values shown were obtained from the 35% *caroli* chimaera. In a 4th animal, the lymph node cells were typed and were found to be 80% *caroli*.

*Values show CTL precursor frequency against each target and 95% confidence interval from 2 independent experiments of the type illustrated in Table 2. One chimaera was used in each experiment. Only *Mus caroli* targets showed significant killing. GPI analysis was not done on these chimaeras.

Table 5. Relative potency of supernatants from decidua of pregnant *Mus caroli* and *Mus musculus*

Source of supernatant (1/100) added to MLC culture	% Suppression of CTL generation	
	C3H anti-BALB/c	C57BL/6 anti-BALB/c
None (medium)	0 ± 3.4	0 ± 3.3
<i>Mus caroli</i> × <i>Mus caroli</i> pregnancy	−2 ± 3.1	38.6 ± 2.6
<i>Mus caroli</i> × <i>Mus caroli</i> pregnancy	9.3 ± 3.5	42.9 ± 3.2
C3H × DBA/2 pregnancy	24.6 ± 3.8*	88.5 ± 0.9*
C3H × DBA/2 pregnancy	17.6 ± 2.5*	79.1 ± 1.3*
C3H × C3H pregnancy	37.1 ± 2.0*	75.2 ± 1.8*

*Significantly greater suppression than by supernatants from pregnant *Mus caroli* ($P < 0.05$; Student's t test). CTL were generated in MLC tissue cultures (C3H + irradiated BALB/c cells (H-2^d) plus 1/100 dilution of supernatant). ^{51}Cr -labelled YAC that bear H-2^d determinants were used and as target cells the spontaneous release was determined from cultures containing medium alone. Titration curves were analysed to determine cytotoxic activity in lytic units and the percentage decrease in yield in presence of test supernatant determined with respect to medium control cultures.

that the *Mus caroli*–*Mus musculus* chimaeras already possess sensitized CTL precursors against *Mus caroli*, it was crucial to determine whether the local active suppression generated by a *Mus caroli* embryo interacting with *Mus caroli* decidua cells was as effective as the suppression associated with a *Mus musculus* embryo interacting with *Mus musculus* decidua. Suppression induced by *Mus caroli* trophoblast interacting with *Mus caroli* decidua in a putatively chimaeric uterus should be able to inhibit CTL of both *musculus* and *caroli* origin. Therefore, the potency of suppression by decidual cell supernatants from *Mus musculus* × *Mus musculus* pregnancies was compared to that elaborated by decidua associated with *Mus caroli* × *Mus caroli* pregnancies. The soluble suppressor activity obtained from the decidua associated with a successful *Mus caroli* in

Fig. 1. Effect of placental cells on lymphokine-activated killer cells. (a) Lysis by *Mus musculus* lymphocyte cultures in IL-2 alone (■) or with *Mus musculus* placental cells (●), or *Mus caroli* placental cells (▲). (b) Lysis by *Mus caroli* splenocytes cultured in IL-2 without (■) or with placental cells (●, ▲). Values are mean $\pm$ s.e.m.

Mus caroli allopregnancy was less potent than that obtained from *Mus musculus* $\times$ *Mus musculus* allopregnancy (Table 5), an observation that was confirmed in two additional experiments. One would predict that a transferred *Mus caroli* embryo to a chimaera would not be expected to create the same magnitude of suppression at the implantation site as would a *Mus musculus* embryo, provided of course, the decidua was not primarily of *Mus caroli* type. This experiment and typing of the decidua of the chimaera has not been done. Figure 1 shows that the ability of placental cells from *Mus caroli* to inhibit LAK activity on YAC target cells was also less than the activity of a *Mus musculus* placental preparation. A similar finding was obtained in a second experiment, indicating that the strength of the local defence system of a *Mus caroli* embryo is inherently less than that of *Mus musculus*. In a chimaera, therefore, a *Mus caroli* embryo would be much less well defended by direct placental suppression and by decidua-associated suppressor activity than would be a *Mus musculus* embryo.

Discussion

Previous studies to test the role of the maternal immune system in the destruction of xenogeneic murine pregnancies have shown that resorption occurs even in putatively immunodeficient mice (i.e. cyclosporine-treated mice, *nu/nu* mice, presumptively tolerant interspecific chimaeras). It was suggested that the xenogeneic embryos succumb due to a biological defect in trophoblast-uterine interaction and that the maternal immune system plays a secondary role in destruction of the fetus (Croy *et al.*, 1985). The results presented in this paper examine certain assumptions about the putatively immunologically unreactive recipient mice upon which previous conclusions were based. The discovery of pre-sensitization of *Mus musculus* against *Mus caroli* antigens explains why embryo destruction may occur so rapidly in *Mus musculus* hosts and why the reaction is not suppressed by cyclosporine A. The explanation for pre-sensitization is unknown but may represent sensitization by a cross-reactive antigen such as a virus that would be seen in association with 'self MHC' as a modified-self (allo- or xeno-antigen).

Athymic *nu/nu* mice are certainly competent to reject certain types of grafted tumours, albeit not by a specific adaptive immune response but due to activation of non-specific effector cells. The *nu/nu* mice clearly may have the ability to discriminate against xenogeneic blastocysts at implantation much earlier in pregnancy than usual and this selective elimination of xenogeneic embryos is consistent with the greater cytotoxic activity of activated macrophages, against xenogeneic target cells (Cabilly & Galliley, 1981). The finding that *nu/nu* mice possess cytotoxic and cytostatic inhibitors of an embryonic placental cell line further indicates that these mice possess an active effector mechanism.

Interspecies chimaeric mice have not been found in our study to be tolerant to unrelated *Mus caroli* and chimaeras possess pre-sensitization against lymphoblast antigens of unrelated *Mus caroli* donor. *Mus caroli* embryos also generate less local suppressor activity even when gestating in a *Mus caroli* uterus. Together, these results suggest that the *Mus caroli* embryo may be particularly susceptible to immune destruction even in the chimaeric uterus. Indeed, when *Mus caroli* $\times$ *Mus musculus* matings are generated in chimaeras such that the mother is truly tolerant of the *Mus caroli* genetic material (because it was her own), live F_1 offspring seem to be produced at a greater than expected number compared to a non-tolerant situation. Only 18 viable hybrids were born from 578 implantations in *Mus musculus* females inseminated with *Mus caroli* spermatozoa (3%); insemination with *Mus musculus* spermatozoa gave 515 newborns per implant (85%) (West, Fredo, Papaioannou, Karr & Chapman, 1977; West, Fredo & Chapman, 1978). Three chimaeric females inseminated with *Mus musculus* spermatozoa produced a total of 114 *Mus musculus* and 15 hybrid offspring in 34 litters (Rossant & Chapman, 1983). If one assumes an 85% success rate for *Mus musculus* implants and an equal number of *Mus musculus* and hybrid blastocysts, then approximately 15/134 (11.2%) *musculus* $\times$ *caroli* blastocysts should have survived (15/134 vs 18/578; $P < 0.005$ by χ^2). Indeed, the proportion of hybrid blastocysts implanting in *Mus musculus* inseminated with *Mus caroli* spermatozoa appears significantly lower on Days 8–14 than the proportion of *Mus musculus* $\times$ *Mus musculus* implants, perhaps due to abnormal and retarded development (West *et al.*, 1977). In a chimaera, therefore, the number of hybrid implantations might be expected to be less than the estimate (134) given above. When hybrids were treated by inseminating chimaeras with *Mus caroli* spermatozoa, for which *Mus caroli* antigens not represented in the *Mus caroli* component of the chimaera would be expected, no successful pregnancies occurred (Rossant & Chapman, 1983). The number of females studied was too small, however, to allow meaningful conclusions.

Although immune reactivity appears to play an important role in the demise of the xenogeneic embryo in the *Mus caroli*–*Mus musculus* system, the exact mechanisms initiating death remain unclear. In the chimaeric uterus, one would expect *Mus caroli* decidual tissue to exist and to interact with *Mus caroli* trophoblast. It is therefore unlikely that *Mus caroli* embryos fail in the chimaeric uterus because of a complete species incompatibility between the decidua and the trophoblast. The hypothesis that *Mus caroli* blastocysts die in the chimaeric uterus because they are more fastidious is difficult to test experimentally. It seems more likely that a species-restricted ability of trophoblast to recruit local active suppressor cells and a lesser recruitment in a *caroli*–*caroli* trophoblast–decidua interaction as compared to *musculus*–*musculus* interaction leaves *caroli* embryos highly susceptible to attack by the sensitized maternal immune system (Clark *et al.*, 1983; Clark & Slapsys, 1985). *Mus musculus* embryos, however, would nevertheless be able to survive much longer in the *Mus caroli* uterus because the magnitude of the immune attack would be less (i.e. *Mus caroli* are not pre-sensitized to *Mus musculus*), and because *Mus musculus* placental cells are more potent inhibitors of *Mus caroli* effectors than are *Mus caroli* placental cells (i.e. *Mus musculus* embryos are intrinsically better defended at the placental level in a *Mus musculus* or *Mus caroli* uterus). One prediction from these considerations is that an immunized female *Mus caroli* should show rapid resorption of a *Mus musculus* embryo inasmuch as local decidua-associated suppressor cell activity should be absent. This experiment is currently being planned. A second hypothesis to explain xenoembryo death proposes that the maternal immune system actually damages the embryo (and

trophoblast) such that it cannot effectively defend itself (i.e. generate local active suppression). Whether the actual mechanism of trophoblast dysfunction is mediated by specific antibodies, specific CTL, or non-specific effectors such as LAK and activated macrophage (both activated by lymphokine produced by activated T cells) is unknown. No data yet exist to test the hypothesis but the concept is none the less worthy of investigation because antigen-specific humoral and cellular immune responses to trophoblast antigens seem to occur in early pregnancy (Youtananukorn, Matankasombut & Osathanondh, 1974; Davies & Browne, 1985) and anti-trophoblast types of immune reactivity may be modifiable to either increase or decrease reproductive success. Since antigens shared by lymphocytes/lymphoblast and trophoblast cells may be involved in these phenomena (McConnachie & McIntyre, 1984), it will be important to test whether the *Mus caroli*-specific CTL generated by incubating *Mus musculus* spleen cells in IL-2 can compromise the function of *Mus caroli* trophoblast cells.

It is important to understand the nature of the balance between immune reactivity and local defence systems that may determine the success or failure of xenoembryo transfer before extensive attempts are made to gestate embryos from endangered species in xenogeneic or chimaeric recipients. It seems unlikely that the *Mus caroli*-*Mus musculus* pair is the only arrangement in which pre-existing hypersensitivity to one parental type occurs. Indeed, our results suggest that testing for such hypersensitivity could predict which xenogeneic blastocyst transfers would be clinically successful.

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