Effects of alterations in the immunocompetent status of *Mus musculus* females on the survival of transferred *Mus caroli* embryos

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Summary. The role of the immune system in promoting the midterm death of *Mus caroli* embryos transferred to the *Mus musculus* uterus was studied *in vivo* by transferring *M. caroli* blastocysts to recipients with altered immune status. Transfers of embryos to chimaeric mothers (*Mus musculus* ↔ *Mus caroli*), which were expected to be tolerant of species antigens, resulted in survival of *M. musculus* embryos but death of *M. caroli* embryos. The preferential survival of *M. musculus* embryos was explained by showing that *M. musculus* embryos can survive in the *M. caroli* uterus. Transfers to T cell-deficient mice of genotype *nu/nu* and to NK cell-deficient mice of genotype *bg/bg* as well as treatment of normal transfer recipients with Cyclosporin A or anti-Ia antiserum failed to prolong survival. However, immunization of recipients with *M. caroli* lymphocytes promoted more rapid and uniform failure of the interspecies pregnancy. Cytotoxic cells were detected in the resorbing embryos on Day 10.5 in immune pregnancies and on Day 12.5 in non-immune pregnancies and these cells were promiscuous in their pattern of lysis, showing equal reactivity against *M. caroli*, transfer recipient and 3rd party target cells. These experiments show that failure of *M. caroli* embryos in the *M. musculus* uterus is complex, but probably does not involve responses by classical cytotoxic T lymphocyte or natural killer cell pathways. Participation of the immune system in the resorption process, however, is confirmed and is associated with generation of promiscuous cytolytic cells.

Introduction

Successful gestation of the semi-allogeneic fetus has remained a paradox in transplantation biology (Beer & Billingham, 1976). To further understanding of the mechanisms normally involved in successful pregnancy, we have developed a rodent embryo-transfer system in which consistent midterm failure of pregnancy occurs with apparent involvement of maternal immune reactivity against the fetus (Croy, Rossant & Clark, 1982). Xenogeneic embryos from females of the mouse species *Mus caroli*, when placed in the uterus of pseudopregnant females of the species *M. musculus*, implant and grow normally until mid-gestation and then resorb. The resorbing embryos are infiltrated by cytolytic cells expressing T-cell lineage markers (CTL). Furthermore, non-specific suppressor cells, usually associated with successful pregnancies, are absent from the decidua of the *M. caroli* conceptuses before death (Clark, Slapsys, Croy & Rossant, 1983). Other experiments have shown that matching the genotype of the trophoblast to that of the foster mother will rescue the *M. caroli* embryos (Rossant, Mauro & Croy, 1982; Rossant, Croy, Clark & Chapman, 1983), suggesting that failure of *M. caroli* trophoblast to recruit suppressor cells may allow access of CTL to the fetus, causing death. None of these experiments proves, however, that absence of suppression and appearance of CTL are the sole factors determining *M. caroli* death. This could

only be proved by successful gestation of *M. caroli* embryos in recipients unable to generate a cytotoxic response against the embryos. We have attempted to define the contribution of the immune system to the death of *M. caroli* embryos by assessing intrauterine viability of *M. caroli* embryos in putatively tolerant, immunodeficient and immunized animals.

Materials and Methods

Mice. The *M. caroli* mice were bred at Brock University as a random stock; *M. musculus* animals included C57BL/6-bg/bg mice, a gift from Dr. R. Swank, Roswell Park Memorial Institute, Buffalo, NY, U.S.A., C3H/HeJ and DBA/2J mice purchased from Jackson Laboratories, Bar Harbor, ME, U.S.A., and CD1 and CD1-nu/nu mice purchased from Charles River Laboratories, St Constant, Quebec. Aggregation and blastocyst injection chimaeras were made between *M. caroli* and *M. musculus* of the CD1 strain as previously described (Rossant *et al.*, 1982) and are designated *M. caroli* ↔ *M. musculus* chimaeras. Transferred *M. musculus* blastocysts were always of the CD1 genotype and CD1 females were used as recipients unless otherwise indicated. All mice were housed under standard conditions except for the nude mice which were caged under filter caps with sterilized food, water and bedding and housed in rooms remote from other animals. Mice were used between 6 and 14 weeks of age.

Immunization of *M. musculus* females. CD1 females, 6 weeks of age, were each injected intraperitoneally (i.p.) with 10^7 irradiated splenocytes from *M. caroli* animals and given booster injections twice at 2-week intervals. After an additional 3 weeks, these mice were used as embryo transfer recipients.

Skin grafting. Female nu/nu and male *M. caroli* ↔ *M. musculus* chimaeras were grafted bilaterally in the thoracic region with tail skin from females of the *M. caroli* and *M. musculus* species. Graft sites were covered with plaster casts for 1 week, then uncovered and scored daily for necrosis and sloughing.

Collection of embryos. Blastocyst stage embryos were collected from the uteri of *M. musculus* females 3.5 days after detection of vaginal plugs following natural matings. The morning of plug detection was designated Day 0.5 of pregnancy. Hormone stimulation was used to obtain blastocysts from *M. caroli* females; 2.5 i.u. PMSG (Sigma, St Louis, MO, U.S.A.) was administered i.p. followed 48 h later by 2.5 i.u. hCG (Sigma). Blastocysts were recovered from the uterus 74–76 h after hCG injection. Mice were killed by cervical dislocation and the uteri dissected and flushed with PB1 medium (Whittingham & Wales, 1969) plus 10% fetal calf serum (FCS) to recover the blastocysts. Blastocysts were transferred to the uteri of pseudopregnant mice within 2 h of collection.

Embryo transfers and laparotomy. All recipient females, except *M. caroli*, were selected for oestrus and placed with vasectomized *M. musculus* males. Embryo transfers were performed 2.5 days after detection of a vaginal plug. *M. caroli* females were given 2.5 i.u. PMSG followed 48 h later by 2.5 i.u. hCG and then paired with vasectomized *M. caroli* males or cervically stimulated by a glass rod for 120–300 sec, 14 h after hCG treatment. Because most *M. caroli* × *M. caroli* matings do not result in vaginal plug formation, it was usually not known if the hormone-primed, paired *M. caroli* females were truly pseudopregnant. *M. caroli* females were used as recipients 50–56 h after hCG.

Recipients were anaesthetized with tribromoethanol (Green, 1975) administered i.p. and 2–7 blastocysts of *M. caroli* or *M. musculus* genotype were placed in each uterine horn. To assess the success of pregnancy, mice were anaesthetized 9–19 days after transfer and laparotomy was performed through a midline abdominal incision. Both uterine horns were exteriorized and examined for the location of viable and resorbing embryos. Pregnancy failure after laparotomy was

difficult to interpret due to the added factors of surgical trauma and anaesthetic depression, but viable *M. musculus* young were born to many recipients after laparotomy.

Cyclosporin A treatment. Cyclosporin A, kindly given by Dr J. F. Borel, Sandoz, Basel, Switzerland, was dissolved in medium-chain triglycerides and administered at doses between 7.5 and 100 mg/kg. Subcutaneous (s.c.) or i.p. injections were given once daily beginning on the day of embryo transfer.

Treatment with anti-Ia^k. Anti-Ia^k monoclonal antibody ascitic fluid was a gift from Dr T. G. Wegmann, University of Alberta (Clone 10-3.6, Salk Cell Distribution Center, San Diego, CA, U.S.A.). C3H/HeJ females that had been successfully mated by vasectomized males were anaesthetized on the morning of plug detection and slowly injected intravenously with ascitic fluid containing 0.5 mg specific IgG. Treated females received embryo transfers 2 days later. Blood samples were collected from the retro-orbital sinus of anaesthetized mice before treatment and at various stages thereafter. These samples were diluted in leucocyte counting fluid and counted, using a haemocytometer, to monitor the effects of the antiserum treatment.

Chromium-51 release assay. Uteri containing resorbing *M. caroli* embryos were dissected from either immune or nonimmune CD1 females and single cell suspensions were prepared by passing the resorbing tissue through a 60-mesh stainless-steel screen into alpha minimum essential media (MEM) (Gibco, Grand Island, NY, U.S.A.) supplemented by 10% FCS, 100 i.u. penicillin/ml, 0.05 mg streptomycin/ml, 10^{-5} M-2-mercaptoethanol and 20 mM-Hepes. The cell suspensions were layered over Lympholyte M (Cedarlane Laboratories, Hornby, Ontario) and centrifuged, and the interface cells were collected, washed and tested for their ability to lyse a variety of ⁵¹Cr-labelled target cells. Target cells included Con-A-induced lymphoblasts of *M. caroli*, CD1, C3H/HeJ and DBA/2J origin, P815 cells or YAC cells. The targets were labelled for 90 min with 0.5 ml Na⁵¹CrO₄ (sp. act. 1 mCi/ml; New England Nuclear, Boston, MA, U.S.A.), washed well and 3×10^3 target cells were co-incubated in 200 μ l supplemented MEM in microtest plate wells with effector cells from the resorbing embryos. Effector:target cell ratios (E:T) were 10:1 or 15:1. Spontaneous release was determined from control cultures containing effector cells derived from age-matched normal CD1 embryos. After incubation for 6 h 100 μ l supernatant were removed from each culture and counted for isotope release in a Philips 4800 series gamma counter. The percentage specific chromium release was determined from the standard formula: (test c.p.m. — spontaneously released c.p.m.)/(NP40 released c.p.m. — spontaneously released c.p.m.) $\times$ 100%.

Results

Embryo transfer to M. caroli females

A series of transfers into *M. caroli* recipients was performed to establish whether (1) *M. caroli* blastocysts could ever survive to term after embryo transfer manipulations and (2) embryo failure was reciprocal between the two species. Embryo transfers to *M. caroli* females were not very successful; 7 pregnancies resulted from a series of 32 transfers (Table 1). Successful pregnancy occurred only in females rendered receptive by pairing to vasectomized *M. caroli* males. Transfers to 3/8 females showing copulatory plugs and 4/16 females not showing plugs resulted in implantation. No implantation occurred in 8/8 females stimulated by a glass rod. In one female, embryos of both species implanted but failed early and this recipient was excluded from the table as she almost certainly represented generalized, non-specific failure to maintain pregnancy. It is clear that *M. caroli* embryos can survive to term after embryo handling and transfer procedures (Mouse 5). This has not been demonstrated previously and shows that failure of *M. caroli* embryos in the *M. musculus* uterus is a biological problem and not a transfer artefact. Also, development of *M. musculus* embryos proceeds further in the *M. caroli* uterus than in the reciprocal transfers; all *M.*

Table 1. Bilateral embryo transfers to *M. caroli* recipients

Recipient number	Gestational age at laparotomy (days)	<i>M. musculus</i> embryos			<i>M. caroli</i> embryos		
		Transferred	Implanted	Status	Transferred	Implanted	Status
1	10.5	4	3	Alive	4	3	Alive
2	11.5	3	1	Alive	3	1	Alive
3	11.5	3	0		3	2	Alive
4	15.5	4	1	Dying	3	0	
5	15.5	3	0		4	4	Live born
6	18.5*	2	1	Dead	0		

* Gestation in *M. caroli* is 17.5 days.

musculus embryos were well formed and showed no signs of resorption. As gestation of *M. caroli* is 17.5 days (Frels, Rossant & Chapman, 1980) it is difficult to know whether the single very large embryo observed in recipient No. 6 on Day 18.5 represents embryonic failure or dystocia. These studies led us to predict that *M. musculus* embryos would survive in interspecific chimaeras, whether or not embryonic failure was mediated by immunological mechanisms.

Transfer of M. caroli and M. musculus blastocysts to female M. caroli ↔ M. musculus chimaeras

If immune rejection is the sole cause of death of *M. caroli* embryos gestating in the *M. musculus* uterus, then transfer of *M. caroli* to immunologically unresponsive hosts would be expected to prolong their survival. We have been unsuccessful in producing xenogenic tolerance to *M. caroli* in *M. musculus* females by using a variety of protocols that do not require host irradiation (Brent, Hansen, Kilshaw & Thomas, 1973; Streilein & Gruchalla, 1981; Chutna, Holan & Hasek, 1981) and therefore elected to produce embryonic chimaeras by blastocyst injection (Rossant & Frels, 1980) or aggregation of morulae (Rossant *et al.*, 1982) and to test any resulting female animals (probability of an XX × XX chimaeric female is 1/4) for their ability to gestate embryos of *M. caroli* and *M. musculus*. Previous studies of intraspecific chimaeras have shown that such animals are tolerant of both component genotypes (Mintz & Silvers, 1967; Phillips & Wegmann, 1973; Matsunaga, Simpson & Meo, 1980). Table 2 reports the results of successful transfers to 9 chimaeric mice. Viable embryos were found in 34/36 *M. musculus* implantation sites examined at laparotomy.

Table 2. Embryo transfer to *M. caroli* ↔ *M. musculus* blastocyst injection and aggregation chimaeras

Recipient number*	% <i>M. caroli</i> coat colour	Gestational age at laparotomy (days)	<i>M. caroli</i> embryos			<i>M. musculus</i> embryos		
			Transferred	Implanted	Live fetuses	Transferred	Implanted	Live fetuses
1†	60	10.5	4	4	2	3	3	3
2	40	12.5	5	5	0	6	6	6
3	55	12.5	7	6	0	6	3	3
4	50	13.5	4	4	1	5	5	3
5	5	14.5	6	6	0	6	5	5
6	70	14.5	7	7	4	6	5	5
7	50	17.5	5	4	0	5	5	5
8	40	19.5	4	4	0	5	4	4
Totals			42	40	7	42	36	34

* An additional *M. caroli* ↔ *M. musculus* aggregation chimaera, 50% *M. caroli* by coat colour, received 11 *M. musculus* embryos only and produced liveborn offspring.

† Aggregation chimaera, all others were blastocyst injection chimaeras.

One litter was liveborn, as predicted. However, amongst 40 *M. caroli* implantation sites only 7 live embryos were found. None of the viable *M. caroli* embryos proceeded to term but we could not determine whether this was a consequence of laparotomy or primary embryo failure. The two animals judged to have the highest percentage of *M. caroli* contribution by coat colour contained the largest number of viable *M. caroli* fetuses. A sufficient number of *M. caroli* embryos resorbing in chimaeras was never available at one time to determine whether cytotoxic cells were actually present in these presumed tolerant recipients and it remains possible that the interspecies chimaeras are not species tolerant, although this seems unlikely.

To estimate the level of tolerance achieved in the interspecies chimaeras, 10 phenotypically male *M. caroli* ↔ *M. musculus* chimaeras were grafted with skin from female *M. caroli* and CD1 mice. Of the 20 grafts, 18 were technically successful and the majority of these failed 2–4 weeks after grafting. Only 1/10 *M. caroli* grafts and 2/8 *M. musculus* grafts were accepted indefinitely. Because the *M. caroli* animals are random bred and grafts exchanged between individuals of this species are generally unsuccessful (B. A. Croy, unpublished) and CD1 mice are also random bred, skin graft rejection probably results from recognition of allogeneic differences. Due to these allogeneic responses, we were unable to demonstrate that successful xenogeneic tolerance had been established. However, recognition of allogeneic differences does not regularly lead to embryonic failure in intraspecies pregnancies and is thus unlikely to explain the loss of *M. caroli* embryos in chimaeras displaying xenogeneic tolerance.

Transfer of M. caroli embryos to immunologically deficient M. musculus females

Transfers to mice receiving Cyclosporin A. An alternative host to one specifically unresponsive to *M. caroli*, in which the role of the immune system can be examined, is one which has a broad immune deficiency. As cytotoxic cells of the T lineage have been detected in resorbing *M. caroli* embryos (Croy *et al.*, 1982), treatment of the embryo transfer recipients with Cyclosporin A, an effective immunosuppressant that predominantly inhibits primary T lymphocyte-dependent immune responses (Bunjes *et al.*, 1982) and is capable of inducing transplantation tolerance across major histocompatibility differences (Borel, Feuer, Magnee & Stahelin, 1977; White, Plumb, Pawelec & Brons, 1979) might prolong survival of *M. caroli* embryos. Six *M. musculus* females received transfers of *M. caroli* embryos to one uterine horn and *M. musculus* embryos to the opposite horn plus various doses of Cyclosporin A beginning on the day of transfer (i.e. 2 days before implantation) and continuing daily thereafter until laparotomy. Low doses of Cyclosporin A failed to prolong the gestation of *M. caroli* embryos while *M. musculus* fetuses remained viable (Table 3). High drug doses adversely affected the survival of all embryos. Embryotoxicity has been reported for comparable doses of Cyclosporin A in the rat and rabbit (Ryffel, 1982). *M. caroli* resorption sites present in animals receiving 7.5 and 15 mg Cyclosporin A/kg were 2–3 times larger than over 500 sites examined in untreated animals. The large resorption sites were black and necrotic and

Table 3. Embryo transfer to *M. musculus* females treated with Cyclosporin A

Cyclosporin A		Gestational age at laparotomy (days)	<i>M. caroli</i> embryos			<i>M. musculus</i> embryos		
Dose (mg/kg)	Route		Transferred	Implanted	Alive	Transferred	Implanted	Alive
7.5	s.c.	11.5	4	4	0	4	4	4
15.0	s.c.	11.5	4	4	0	4	4	3
22.5	s.c.	11.5	5	4	0	5	5	4
22.5	i.p.	11.5	4	4	0	5	5	2
50.0	s.c.	12.5	6	6	0	4	4	0
100.0	s.c.	11.5	5	5	0	4	4	0

contained no evidence of organized embryonic tissue, suggesting that some impairment of the embryo resorption process occurred in the presence of the drug. Inhibition of immune responses by Cyclosporin A treatment, however, could not prevent failure of *M. caroli* embryos.

Transfer of M. caroli embryos to recipients treated with anti-Ia^k antiserum. As a variety of inflammatory cells are associated with T cells in rejecting allografts (Ascher, Ferguson, Hoffman & Simmons, 1979), we also depleted lymphocytes in a less class-specific manner by treatment of embryo transfer recipients with anti-Ia antiserum. It is known that Ia is expressed on B cells and macrophages as well as on some T cells (McDevitt, Delovitch & Press, 1977) and that treatment with anti-Ia antiserum *in vivo* can inhibit macrophage-T cell interactions during antigen presentation (Perry & Green, 1982), deplete B cells from the circulation and lymphoid tissues (Waldor *et al.*, 1984) and promote semiallogeneic bone marrow graft success in non-irradiated recipients (Francescutti, Gambel & Wegmann, 1983). C3H/HeJ females were given anti-Ia^k antibody on Day 0.5 of pseudopregnancy and received bilateral transfers of C3H and *M. caroli* embryos on Day 2.5. Antiserum treatment severely depleted the circulating leucocyte pool and, even by term, the mice had less than one third of their normal leucocyte count; yet no *M. caroli* embryos survived to Day 12.5 of pregnancy (Table 4). The effects of antiserum treatment, however, are compatible with successful gestation as liveborn C3H offspring were obtained. Therefore, the lymphopenia associated with infusion of anti-Ia antiserum does not prolong gestation of *M. caroli* embryos in the *M. musculus* uterus.

Table 4. Embryo transfer to C3H females treated with anti-Ia^k antibody

Recipient number	<i>M. caroli</i> embryos			C3H embryos			% leucocyte decrease*		
	Transferred	Implanted	Alive†	Transferred	Implanted	Alive	Day 2	Day 12	Day 21
1	7	2	0	4	4	4‡	88.3	83.9	66.6
2	7	2	0	4	4	3§	95.1	64.2	63.4

* Leucocyte count on Day 0, before infusion with anti-Ia^k antibody, used for normalizations.

† Both recipients were examined by laparotomy on Day 12.5 of gestation.

‡ 3 full-term offspring born.

§ Aborted on Day 18 of gestation, after laparotomy.

Transfer of M. caroli embryos to genetically immunodeficient mice. Broad immunodeficiencies can result from specific genetic mutations as well as from pharmacological intervention. Mice of the *nu/nu* genotype are athymic (Pantelouris, 1968) and unable to reject many types of allografts and xenografts (Manning, Reed & Schaffer, 1973). *M. caroli* embryos transferred to such mice should therefore not be infiltrated by cytotoxic T cells and the pattern of embryo survival should establish whether such an occurrence is necessary for failure of the xenogeneic pregnancies. The results of a series of such transfers are presented in Table 5 and show that survival of *M. caroli* embryos was not prolonged following implantation in the T cell-deficient mice.

To assess the level of impairment of *nu/nu* mice to *M. caroli* xenoantigens, 7 *nu/nu* females were grafted with tail skin from *M. caroli* females. All 7 grafts survived indefinitely (30–45 days) until the mice were killed for other experiments, which suggests that *nu/nu* mice have no immune effector mechanism to deal with tissue grafts of *M. caroli* origin. It remains possible, however, that the mechanisms for skin graft rejection and embryo rejection differ and that *nu/nu* mice are competent in the latter. To sustain this view, it is necessary to postulate that neither cytolytic T lymphocytes nor DTH-mediated responses effect the death of *M. caroli* embryos, but that other defence mechanisms present in *nu/nu* mice, such as NK cells, antibody-mediated responses or activated macrophages (Kindred, 1979; Sharp & Colston, 1984) are important. Difficulties in obtaining

Table 5. Transfer of *M. caroli* embryos to genetically immunodeficient recipients

Recipient genotype	Gestational age at laparotomy (days)	<i>M. caroli</i> embryos			<i>M. musculus</i> embryos		
		Transferred	Implanted	Alive	Transferred	Implanted	Alive
<i>nu/nu</i>	12.5	10	4	0			
<i>nu/nu</i>	12.5	6	6	0	5	3	3
<i>nu/nu</i>	19.5	4	1	0	4	4	4
<i>nu/nu</i>	Term	4	4	0	5	4	4
<i>nu/nu</i>	Term	4	2	0	5	5	5
<i>bg/bg</i>	11.5	5	1	0			
<i>bg/bg</i>	11.5	4	1	0			
<i>bg/bg</i>	13.5	5	3	0			

regular oestrous cycles in *nu/nu* mice and in maintaining successful pregnancies of transferred *M. musculus* embryos in these recipients even with progesterone supplementation and ovarian transplantation (B. A. Croy, unpublished) have limited our studies of these possible mechanisms.

To test for the role of NK cells in mediating death of *M. caroli* embryos, we transferred *M. caroli* blastocysts to recipients of *bg/bg* genotype. Beige mice are known to have a complete and selective deficiency in NK cells, but their other forms of cell-mediated immunity appear completely normal (Roder & Duwe, 1979). The immune deficiency of these recipients was confirmed by demonstrating that their spleen cells were not able to lyse YAC target cells (data not shown). These embryo transfers failed (Table 5) suggesting that NK cells are unlikely to play a major role in initiating the death of *M. caroli* embryos in the *M. musculus* uterus.

Immunization of recipients does alter the pattern of M. caroli embryo survival

As we had been unable to prolong survival of *M. caroli* embryos in immunologically impaired animals, we considered whether prior immunization of mice with *M. caroli* lymphoid tissue would alter the pattern of survival of *M. caroli* embryos after transfer. *M. musculus* (CD1) females received 3 doses of *M. caroli* splenocytes 2 weeks apart before being used as embryo transfer recipients. Transfer of *M. caroli* embryos to immune recipients significantly changed the resorption pattern (Text-fig. 1). All embryos were resorbed by Day 10.5 of gestation, eliminating the variability in both the frequency and the timing of resorption seen in non-immune females. Immunization therefore resulted in a more rapid and more consistent failure of *M. caroli* embryos. These results demonstrate that the immune system is indeed an active participant in the death and resorption process following xenogeneic embryo transfer in the mouse.

Cytotoxic cells are present in M. caroli embryos resorbing in immunized mice

In a previous series of experiments, we reported that cytotoxic cells could be isolated from *M. caroli* embryos resorbing in unimmunized *M. musculus* between 11.5 and 13.5 days of gestation, with the peak of reactivity against *M. caroli* Con A lymphoblast targets occurring on Day 12.5 (Croy *et al.*, 1982). To confirm participation of the immune system in the death of *M. caroli* embryos in immunized mice, we examined cells isolated earlier in pregnancy for their ability to lyse *M. caroli*, CD1 and 3rd party lymphoblast targets. Cells recovered on Day 10.5 of gestation from embryos resorbing in immune recipients were cytotoxic to *M. caroli* targets (Table 6), whereas cells isolated at this time from non-immune recipients were not (Croy *et al.*, 1982). The cells isolated from immune mice were equally cytotoxic for target cells of transfer recipient or 3rd party genotypes. Lack of specificity for the presenting antigens is not shown by CD1 spleen cells sensitized to *M. caroli* cells *in vitro* (Croy, Rossant & Clark, 1984) and this suggests that the

Text-fig. 1. Resorption of *M. caroli* embryos in non-immune (open bars) and immune (hatched bar) recipients. Numbers in parentheses represent the total number of implantation sites examined at each time point.

Table 6. Specificity of cytotoxic cells isolated from resorbing *M. caroli* embryos

Gestational age (days)	Status of recipient	E:T ratio	% ⁵¹ Cr Release on target:*					
			<i>M. caroli</i> blasts	C3H blasts	CD1 blasts	P815	DBA blasts	YAC cells
10.5	Immune	15:1	7.2 ± 0.7	9.9 ± 0.7	8.4 ± 0.6	—	—	—
12.5	Non-immune	10:1	8.5 ± 0.8	7.3 ± 0.4	—	12.3 ± 0.5	—	—
12.5	Non-immune	15:1	8.7 ± 2.2	—	—	8.7 ± 1.4	7.3 ± 1.3	8.7 ± 0.7

* Values are mean ± s.e.m., n = 3 for cells from immunized mice and n = 6 for cells from non-immune mice.

cytotoxic T cells present in resorbing embryos are not classical CTL but may be a novel cell class or an unusual mixture of cytotoxic cells.

The unusual pattern of lysis displayed by cells isolated from embryos resorbing in immune females could have resulted from the immunizations or may occur in cells isolated from embryos resorbing in unmanipulated females. Therefore, *M. caroli* blastocysts were transferred to non-immune CD1 recipients and cells were isolated from the resorbing embryos on Day 12.5 of gestation and assayed for cytotoxic specificity (Table 6). In two similar experiments we again demonstrated cells with a promiscuous pattern of cytotoxicity showing low but significant levels of cytotoxic activity against all targets. The absence of preferential lysis of the *M. caroli* targets in non-immune and immune transfer recipients suggests that an identical cytotoxic process, not involving classical CTL, is occurring in embryos resorbing in both classes of recipients. Also, immunization can advance the time of appearance of these cytotoxic cells, but cannot significantly alter their pattern or level of reactivity.

Discussion

This series of in-vivo experiments was performed to elucidate the role of the immune system in the midterm pregnancy failure of *M. caroli* embryos in the *M. musculus* uterus. The ideal hosts in which to test the hypothesis that immune responses directed specifically against *M. caroli* are the sole cause of death of *M. caroli* embryos would be females specifically unresponsive to *M. caroli* antigens, i.e. tolerant recipients. In such hosts cytotoxic responses to *M. caroli* antigens would not occur but all other immune, hormonal and physiological responses of pregnancy would remain normal. The procedure we employed to obtain xenogeneic tolerance was the production of embryonic chimaeras (Mintz & Silvers, 1967; Phillips & Wegmann, 1973; Matsunaga *et al.*, 1980). Such *M. musculus* $\leftrightarrow$ *M. caroli* chimaeras are viable and resemble allogeneic chimaeras in all properties examined to date (Rossant & Chapman, 1983). Therefore, if immune responses directed against species antigens are important in the death of *M. caroli* embryos, *M. caroli* fetuses should survive in these recipients. However, *M. caroli* embryo failure was observed, leading to the conclusion that immune rejection is not the entire explanation for death of *M. caroli* embryos in either the chimaeric or *M. musculus* uterus. We were unable to demonstrate experimentally by skin grafting or by MLR and CTL assays (B. A. Croy, unpublished) that these chimaeras were actually species tolerant due to allogeneic variability within each of the parental species.

We also performed a series of transfers to immunodeficient recipients to determine whether survival of *M. caroli* embryos could be prolonged by selective depletion of immunocompetent cell populations. We observed a preferential loss of *M. caroli* fetuses over *M. musculus* fetuses in all of the immunodeficient recipients, and the time of death and gross pathological appearance of the resorbing embryos resembled that of *M. caroli* embryo failure in the uterus of normal *M. musculus* females. The necessity for participation of classic CTL effectors in *M. caroli* embryo death was eliminated by failure of *M. caroli* embryos in Cyclosporin A-treated recipients (Bunjes *et al.*, 1982) and in *nu/nu* recipients (Kindred, 1979). Death of *M. caroli* embryos in *nu/nu* mice also eliminated any essential effector role for DTH responses (Kindred, 1979) while their death in *bg/bg* mice eliminated NK cells as a principal effector mechanism (Roder & Duwe, 1979). T cell-dependent antibody-mediated B cell responses are significantly reduced in *nu/nu* mice (Croy & Osoba, 1973), mice treated with Cyclosporin A (Bunjes *et al.*, 1982) and mice treated with anti-Ia antibody (Waldor *et al.*, 1984), making antibody-mediated embryonic failure unlikely. The only classes of immune effector cells not substantially reduced in any of the immunodeficient recipients were the macrophage and NC lineages (Stutman & Cuttito, 1981), leaving open the possibility that these cells, which are not part of the specific immune effector system, may cause embryonic death. It is also still formally possible that failure of *M. caroli* embryos was mediated by different graft-destroying mechanisms in each of the various immunodeficient hosts. We have therefore been unable to demonstrate in immunodeficient *M. musculus* and *M. caroli* $\leftrightarrow$ *M. musculus* chimaeric recipients that immune responses directed against *M. caroli* fetuses are the sole cause of pregnancy failure.

Immunization of recipients with *M. caroli* lymphocytes advanced both the time of *M. caroli* embryo death and the time of appearance of cytotoxic cells of the same non-specific type as found in non-immune recipients (Table 6). This indicated a clear involvement of the immune system in the actual process of destroying and resorbing the *M. caroli* fetuses. This was also illustrated by persistence of large, necrotic *M. caroli* fetuses in the recipients receiving Cyclosporin A. However, since fetuses still die in immunocompromised recipients, access of immune effector cells to the fetus may be a secondary consequence of some primary non-immunological failure.

If immunological events do not initiate failure of *M. caroli* embryos, what is the principal element determining their fate? Success or failure of *M. caroli* $\leftrightarrow$ *M. musculus* chimaeras in the *M. musculus* uterus was previously shown to be determined by the genotype of trophoblast. *M. musculus* trophoblast allowed chimaeric (Rossant *et al.*, 1982) and fully xenogenic fetuses (Rossant *et al.*, 1983) to proceed to term in the *M. musculus* uterus. In this report, *M. caroli* embryos survived

longest in chimaeras having the greatest *M. caroli* contribution (Table 2). That is, prolonged survival of *M. caroli* embryos was seen in the females in which *M. caroli* trophoblast would have the highest probability of matching with uterine cells of its own genotype at the implantation site. The results of transfers to chimaeras are thus consistent with the suggestion that the primary event in *M. caroli* embryo failure is incorrect interaction between *M. caroli* trophoblast and *M. musculus* uterine tissue.

This unsuccessful interaction may lead both to failure of growth and development of the xenogeneic *M. caroli* conceptuses and to failure of regulation of immunocompetent cells within the *M. musculus* uterus. Inadequate placental growth and function resulting from failure of trophoblast-uterine interactions would lead to fetal death from nutritional deficiency in immunodeficient and normal mice. Unsuccessful interaction between *M. caroli* trophoblast and *M. musculus* uterine tissue may also, however, lead to alteration in the immune status of the uterine environment. We have already shown that local suppressor cells are deficient at the sites of *M. caroli* embryo implantation in the *M. musculus* uterus (Clark *et al.*, 1983). In normal or immunized recipients, embryonic failure would then be mediated or hastened by unregulated immune effector cells gaining access to the conceptus. Our current studies are directed towards definition of the defective interactions between *M. caroli* trophoblast and the *M. musculus* uterus.

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