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Immunological Aspects of Gestation in the Tammar Wallaby, *Macropus eugenii*

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Abstract

Sensitization to male histocompatibility antigens and repeated pregnancy to the same male were found to have little effect on fertility or length of gestation in the tammar wallaby, *M. eugenii*. However, in some sensitized females a long interval occurred between removal of pouch young and the next birth. In addition to studies on fertility, the immunological response of female tammars to their mate has been examined by one-way mixed leucocyte culture (MLC) carried out at the beginning and end of one breeding season. In virgin females, examined at the beginning of the breeding season, the MLC response to the prospective mate peaked on day 6. In contrast, by the end of the season, MLC responses were much lower and peaked earlier, on day 3 to day 5.

Introduction

In eutherian pregnancy the foetus is exempted from allograft rejection. Exactly how this occurs is uncertain, although the prevailing view is that the trophoblast provides an immunological barrier between mother and foetus which prevents rejection (Beer and Billingham 1971; Wynn 1971). One way in which the trophoblast may carry out this role is by local secretion of specific gestational hormones capable of suppressing immune responses between mother and foetus (Amoroso and Perry 1975).

In marsupials the period of foeto–maternal contact is very short. Foetal tissues are in close apposition with the uterine endometrium for only 3–8 days (Hughes 1974) when most of embryogenesis takes place. This stage is preceded by an unattached period of variable length when the partially developed blastocyst floats freely in the uterus, surrounded by a keratinous shell of maternal origin (Hughes 1974).

Since the period of close foeto–maternal contact in marsupials is so short, it has been suggested (Tyndale-Biscoe 1973; Moors 1974; Lillegraven 1975) that marsupials may not have evolved mechanisms similar to those that protect the eutherian foetus from rejection and allow a prolonged intra-uterine development. If this is so, it seems possible that sensitization of the female to male histocompatibility antigens may adversely affect fertility in marsupials. In eutherians sensitization appears to have little effect on fertility or the length of gestation (Beer and Billingham 1971).

The tammar wallaby, *Macropus eugenii*, provided a suitable species in which to examine this question. Tammar females undergo a succession of 28-day oestrous cycles from February until June when they enter a period of seasonal quiescence. Since the length of gestation is about one day shorter than the oestrous cycle, if

females are run with a male and young are removed after birth, a series of up to five pregnancies can occur before seasonal quiescence supervenes. Normally the presence of a suckling young prevents another birth (Tyndale-Biscoe *et al.* 1974). The successive removal of pouch young in this manner allowed us to examine the effects of repeated pregnancy to the same male on parity and on the length of gestation. We also looked for changes in immunological reactivity by examining the response of female peripheral blood leucocytes to male cells in one-way mixed leucocyte culture (MLC). Some female tammars were also sensitized to their male mate with skin grafts and the effects of sensitization on fertility and MLC responses were examined in the next breeding season.

Methods

Animals

All animals used came from the established colony of outbred tammar wallabies, maintained at the Division of Wildlife Research, CSIRO, Gungahlin, A.C.T., which are descended from stock derived from Kangaroo Island, S.A. In addition tammars from Garden Island, W.A., were used. It is estimated that these two island races have been in geographical isolation for at least 7000 years (Main 1961).

Female tammars were divided into four experimental groups which were treated according to a similar protocol. During seasonal quiescence the MLC response of each female was tested against her prospective mate for the next season and against another male of the same race. This response will be referred to as the first MLC response. Three females in group 1 and half of the females in each of the other groups then received a skin graft from the prospective mate. When the grafts had been rejected, breeding groups were established for the next season. Each mated group was kept in a triangular yard where the animals were readily caught with long handled nets. Females were examined for pouch young at least once a week, or daily if a birth was imminent, and young were removed from the pouch 7–10 days after birth. The length of gestation has been estimated as the interval between the removal of pouch young on day 0 and the subsequent birth. At the end of the breeding season all females were again tested by MLC for reactivity to their mate and to the same 'non-mated' male. This response will be referred to as the second MLC response.

Group 1 comprised seven Kangaroo Island (K.I.) females that were successively mated to the same K.I. male for 4 years. For the first 2 years breeding records were kept and young were removed 1 or 2 days after birth. In the third and fourth years the young were not removed so that each female produced one offspring by the same male. Before the onset of the fifth breeding season the three surviving females were tested in MLC and given skin grafts from the male mate. Only two of these females survived until the end of the fifth breeding season.

Group 2 consisted of 12 K.I. females which were isolated before their first oestrus and mated in groups of four to a K.I. male. One female in this group died and was replaced by a young post-lactational female (No. 3809).

Group 3 consisted of four K.I. females mated to a Garden Island (G.I.) male whilst group 4 consisted of four G.I. females mated to a K.I. male. Females used in groups 3 and 4 were post-lactational females, isolated after breeding with males of their own race. All males used were mature animals taken from the general breeding colony.

Skin Grafting

Animals to be skin grafted received premedication with 25 mg Themalon (diethylthiambutene) intravenously in the lateral tail vein, followed by anaesthesia with 2–3 ml Surital (sodium amital, 4% w/v in 0·9% saline). Additional Surital was given as required. Skin from the male donor was removed in a 1 by 8-cm strip from the belly. Three 10 by 20-mm squares of donor skin were grafted onto sites on the base of the tail of recipient females according to methods described by Billingham (1961) for full thickness skin grafts. The site was dressed with Tulle Gras and wrapped firmly with Leucoplast elastic bandage. Grafts were examined and dressings changed daily. Graft survival time was estimated macroscopically as the interval until the graft began to desiccate and lift away from the underlying dermis. Biopsies were removed and fixed for histological examination.

Mixed Leucocyte Culture

MLC was carried out according to methods described by Lafferty *et al.* (1974). Heparinized blood (15–25 ml) was removed from a lateral tail vein and peripheral blood leucocytes were separated from red cells on an isopaque-ficoll gradient (Parish and Hayward 1974). The cells were washed twice and made up to the required density in Eagle's minimal essential medium (Grand Island Co., F15) containing 2-mercaptoethanol at a final concentration of 10^{-5} M and supplemented with 10% foetal calf serum, penicillin (100 μg/ml) and streptomycin (100 μg/ml). Stimulating cell populations were made up at 1×10^7 cells/ml and irradiated with 1200 R from a ^{60}Co source at a rate of 280 rad/min. Cell cultures were prepared as mixtures of equal volumes of reacting and stimulating cells, each at an initial cell density of 2×10^6 cells/ml. Aliquots of 0·2 ml were added to each of 32 replicate cups in Microtest II 96-well tissue culture plates (Falcon 3040) and cultured in a sealed box at 37°C in an atmosphere of 10% CO_2, 7% O_2 and 83% N_2. At daily intervals 3 μCi of [^{3}H]thymidine (Amersham, TRA 120, specific activity 5 Ci/mmol) were added to four of the 32 wells. Five hours later cells from labelled wells were collected on glass fibre discs, using an automatic cell sample harvester (M.A.S.H. II, Microbiological Associates). The discs were placed in glass vials with 8 ml scintillation fluid (0·5% 2,5-diphenyloxazole in toluene) and the amount of [^{3}H]thymidine incorporation was measured in a liquid scintillation counter. Results are given as mean cpm of replicate wells $\pm$ s.e. Some difficulty was experienced in collecting sufficiently large blood samples from the smallest female wallabies. To conserve female peripheral blood leucocytes the response of male peripheral blood leucocytes to autologous irradiated cells was used as the control, since preliminary experiments showed that this response closely resembled that of female peripheral blood leucocytes with autologous irradiated cells, with very low levels of [^{3}H]thymidine uptake throughout the culture period. In each case a stimulation index (S.I.) was calculated for the peak of the response as

$$\text{S.I.} = \frac{\text{cpm female response to male}}{\text{cpm control response}}.$$

Results

Skin Graft Rejection

The multiparous K.I. females of group 1 rejected skin grafts from their K.I. mate within 14–16 days. Females of group 2, which were virgin when grafting took place, rejected K.I. skin grafts within 10–13 days. The difference in mean survival time for grafts in groups 1 and 2 was not statistically significant. K.I. females of group 3 rejected skin grafts from a G.I. male in 8 days, whereas G.I. females of group 4 rejected skin grafts from a K.I. male in 11 days.

Fertility and Length of Gestation

Despite repeated pregnancies to the same male the females in group 1 showed no change in number of pregnancies, gestation length or neonatal weight between the first and second year (Table 1). Even in the fifth breeding season the two females which had been sensitized to the male by skin grafting showed no significant difference from the first year in mean length of gestation. The values obtained for the first and second breeding season correspond closely with the mean gestation time of 27·5 days obtained by Berger (1970) whilst the mean length of gestation in the fifth breeding season was one day longer (28·6 days). Although skin grafting seemed to have little effect on the mean length of gestation in multiparous females, four births to four different skin-grafted females in group 2 were delayed for long intervals after removal of pouch young (Table 2), which approximated two or three times the normal length of gestation. Nevertheless, skin grafting did not affect the total number of births to a female in one breeding season, since sensitized females continued to breed for a longer period than normal females.

Only four births were recorded to females of groups 3 and 4, which had been put with males of the other island race. As the females chosen for these breeding groups were young post-lactational females isolated from general breeding stock, these four births can be attributed to matings from the previous breeding season when the females had been kept with males of similar race. Since removal of pouch young was not followed by another birth, there was either no successful mating between animals of different races at post-partum oestrus, or a subsequent pregnancy was not successfully completed.

Table 1. Live births, length of gestation and neonatal weight in tammar wallabies of group 1—K.I. females mated to the same K.I. male for five successive breeding seasons

Each female raised a single offspring in 1973 and 1974. The values for 1975 are from females which had received grafts of skin from their male mate before the onset of the breeding season. Results are presented as mean±s.d. The number is shown in parentheses

Breeding season	Fertility (births per female)	Length of gestation (days)	Neonatal weight (mg)
1971	2·7±1·1 (7)	27·8±1·2 (10)[A]	413·5±54·7 (13)
1972	3·3±1·0 (7)	27·7±0·75 (13)	409·6±42·8 (14)
1975	3·5±0·7 (2)	28·6±1·14 (5)	—

[A]Length of first gestation of the season cannot be determined because it results from a reactivated blastocyst of the previous season.

Mixed Leucocyte Culture

The reactivity of females in MLC against their mate and another unrelated male was examined at the beginning and end of one breeding season. Stimulation indices for these responses are given in Table 3.

Table 2. Live births and length of gestation in tammar wallabies of group 2—K.I. females mated to a K.I. male

Six females of this group received skin grafts from their prospective mate before the onset of the breeding season. Results are presented as mean±s.d. The number is shown in parentheses

Treatment	Fertility (births per female)	Length of gestation (days)	Interval (days)
Non-grafted	2·7±1·2 (6)	27·3±3·47 (10)	24, 25, 25, 26, 26, 26, 27, 29, 29, 36
Grafted	2·3±1·0 (6)	49·1±25·3 (8)	26, 28, 29, 31, 49, 60, 79, 91

The most extensive data were obtained for females of group 2 which were virgin at the time the first MLC was carried out. Stimulation indices for the first MLC varied widely between individuals (range 2·4–44·0) but there was no evidence that the females responded any better to the prospective mate than to the unrelated male. Stimulation indices against the prospective mate were higher in only 7 of 12 cases, which is as expected if the mate had been chosen at random. A typical time course

for [^{3}H]thymidine uptake in the first MLC is shown in Fig. 1. The response was monophasic and reached a peak on day 6 or day 7.

A second MLC was carried out on 8 of the 12 females of group 2 at the end of the breeding season. The stimulation indices were often lower in this second MLC than they had been previously (Table 3), both in the response with the mate and in the response with the unrelated male. The depression in MLC reactivity occurred both in skin-grafted and in non-skin-grafted females. Fig. 2 shows a typical time course for [^{3}H]thymidine uptake in the second MLC.

Table 3. Stimulation indices (S.I.) for MLC responses between female tammars and their mate or another non-mated male

The first MLC response (MLC-1) was carried out at the beginning of the breeding season and the second (MLC-2) was carried out at the end of the breeding season

Group	S.I. against male mate		S.I. against non-mated male	
	MLC-1	MLC-2	MLC-1	MLC-2
1	6·1	1·4[A]	14·6	0·5[A]
	12·5	24·0[A]	24·2	4·7[A]
2	13·9	13·0	33·0	2·8
	11·3	11·8	39·7	23·3
	5·7	3·4	4·9	46·2
	28·8	0·8	27·4	2·5
	8·0	—	4·0	—
	16·5	—	11·1	—
	10·3	3·0[A]	14·3	2·7[A]
	15·9	2·0[A]	16·5	2·8[A]
	18·2	7·2[A]	5·7	2·0[A]
	4·5[B]	2·1[A]	11·6	8·0[A]
	2·9	—	2·4	—
	44·0	—	19·0	—
3	13·0	4·0	3·2	—
	10·0	3·7	1·3	—
	11·2	1·5[A]	2·2	—
	18·3	3·5[A]	8·0	—
4	0·9	10·2	1·0	10·2
	1·4	5·2	1·1	17·6
	0·7	0·5[A]	0·9	2·1[A]
	1·1	—	1·5	—

[A] Response after skin grafting from male mate.
[B] Results from female 3809.

The second MLC response against the male mate was typically biphasic, with an early peak of [^{3}H]thymidine uptake occurring on days 3, 4 or 5 and a second, lower peak occurring on days 6 or 7. In contrast, the response against the unrelated male peaked before day 6 in only two of the eight females tested. (One example of an early response to an unrelated male is shown in Fig. 2.) These differences of timing in the peak of the response for the first and second MLC are illustrated in Fig. 3.

The two surviving multiparous females of group 1 were tested for MLC reactivity at the beginning and end of their fifth breeding season. Stimulation indices for these

responses are also given in Table 3. In the first MLC both females reacted more vigorously against the unrelated male than against their mate. However, the peak of the response against the mate occurred on day 5 in each case, one day earlier than the peak of the response against the unrelated male. When the second MLC was

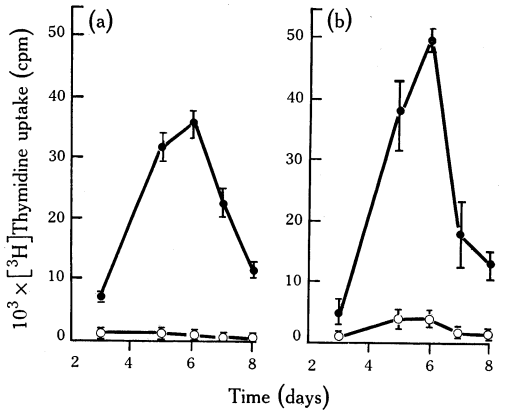

Fig. 1. MLC response of a virgin female K.I. tammar from group 2 at the beginning of the breeding season with (*a*) prospective K.I. male, and (*b*) an unrelated K.I. male. The response is shown by [^{3}H]thymidine uptake in experimental (●) and control (○) cultures.

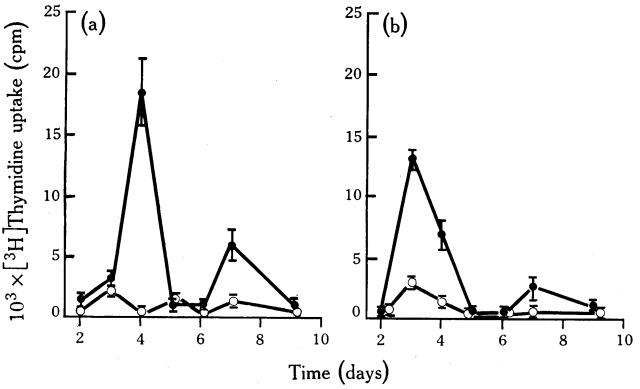

Fig. 2. MLC response of a female K.I. tammar from group 2 at the end of the first breeding season with (*a*) the K.I. male mate, and (*b*) an unrelated K.I. male. The response is shown by [^{3}H]thymidine uptake in experimental (●) and control (○) cultures.

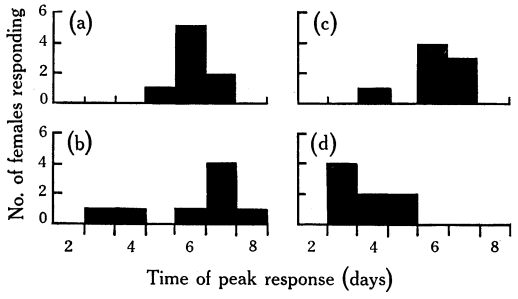

Fig. 3. Timing of the peak of [^{3}H]thymidine uptake in MLC responses of female tammars from groups 1 and 2. The histograms show the response to an unrelated K.I. male at (*a*) the beginning and (*b*) the end of the breeding season, and the response to the male mate at (*c*) the beginning and (*d*) the end of the breeding season. The difference between (*c*) and (*d*) is statistically significant ($\chi^2 = 15{\cdot}73$, d.f. $= 7$, $P < 0{\cdot}05$).

carried out both females had rejected skin grafts from their mate and had given birth to three or four more young. One female now gave little or no MLC response to both males, whilst the second female reacted poorly against the unrelated male but strongly against her mate. This strong response to the mate again peaked early, on day 4.

Table 3 also gives stimulation indices for MLC responses for the K.I. and G.I. females of groups 3 and 4 which were mated to males of the other race. The first MLC responses for females of group 3 (K.I. females mated to a G.I. male) resembled those obtained in group 2 (K.I. females mated to a K.I. male) in that the peak of [^{3}H]thymidine uptake occurred on day 6 or day 7. However, in the second MLC the response was very low, both in normal and skin-grafted females.

G.I. females mated to a K.I. male gave a poor response to both K.I. males in their first MLC, although when tested against a non-related G.I. male they responded vigorously (stimulation indices 4·0–14·2). The two normal non-grafted females reacted more strongly to the K.I. males in the second MLC but the response of a skin-grafted female still remained very low.

Discussion

Female eutherians can be mated successively to the same male with no effects on fertility or on the length of gestation even after sensitization to male histocompatibility antigens (Beer and Billingham 1971). This also seems to be the case in tammar wallabies. No significant change in fertility or length of gestation was observed when female tammars were mated successively to the same male for up to 5 years. Also when multiparous females were sensitized to their mate with skin grafts, fertility in the next breeding season was unaffected. On the other hand, four females sensitized to a male while still virgin exhibited a long interval between removal of pouch young and the next birth. However, as they continued to breed for a longer period than normal females this did not affect the total number of births per female. The long intervals between removal of pouch young and birth were approximately two or three times the normal length of gestation. It therefore seems that these females either failed to conceive, perhaps from a reaction to male sperm, or that the first pregnancy after removal of pouch young failed to be maintained to full term. A successful conception then followed at the next or a succeeding oestrus.

The effects of sensitization and repeated pregnancy to one male on the imunological reactivity of female tammars was also investigated. Although the kinetics of the response were similar in all non-mated females, stimulation indices at the peak of the response were quite variable. Ashman *et al.* (1972) have described MLC responses in the tammar and quokka which had similar kinetics if foetal calf serum rather than quokka serum was used to supplement the culture medium. Also, Osoba and Falk (1974) observed that in MLC responses with human peripheral blood leucocytes the level of the response often varied markedly between individuals although the time course of the response remained the same.

The response of female K.I. tammars in MLC against male peripheral blood leucocytes had altered by the end of the breeding season. Stimulation indices were generally lower and the kinetics of the response had also changed. The first MLC response was typically monophasic and reached a peak on day 6 or day 7, whereas in the second MLC against the male mate the peak of [^{3}H]thymidine uptake occurred on days 3, 4 or 5. In the second MLC response with the non-mated male the peak occurred on day 6 or day 7, as at the start of the season.

Similar changes in the kinetics of MLC responses have been described in sensitized animals of various eutherian species, including rats (Wilson and Nowell 1971) and humans (Bondevik and Thorsby 1974). In each case sensitized cells proliferated earlier

in MLC, although the level of the peak response was similar to or even lower than the same MLC response in a normal cell population. In tammars the early peak response in the second MLC occurred both in mated and in skin-grafted females. It therefore seems that female tammars may become sensitized to male histocompatibility antigens during pregnancy, although this does not appear to affect their reproductive efficiency. In this respect also, they resemble eutherians (Beer and Billingham 1971; Maroni and Parrott 1973).

The stimulation indices for responses in the second MLC were often lower than in the first MLC, especially in skin-grafted females. This depression in the level of the response occurred both in reactions with the mate and in reactions with a non-mated male. It may thus reflect a general depression of cell-mediated immunity in sensitized females at the end of the breeding season. However, since observations were not made on normal non-mated females it is possible that these changes are due to a seasonal variation rather than to sensitization. Nevertheless, it is of interest that a similar depression in cell-mediated immunity may also occur in eutherian females. Parmiani and Invernezzi (1975) found that this immunity declines in female mice after multiple pregnancies to a syngeneic male. There is also evidence that both MLC responses and blast transformation in response to phytohaemagglutinin may be depressed during pregnancy (Finn *et al.* 1972; Purtilo *et al.* 1972; St. Hill *et al.* 1973), perhaps due to serum factors (Kasakura 1971; Gatti *et al.* 1973) or the action of gestational hormones (Contractor and Davies 1973).

Attempts were made to interbreed K.I. and G.I. tammars but no hybrid young were born to females in either of the two reciprocal crosses. Despite the absence of hybrid pouch young, the MLC responses of the K.I. females associated with a G.I. male resembled those already described in K.I. females mated with a male of their own race, since stimulation indices were depressed at the end of the breeding season. It is not known whether these K.I. females actually mated with the G.I. male, and thus it is not clear whether the changes in MLC reactivity can be ascribed to the effects of a non-completed pregnancy or whether they are merely the result of seasonal changes in female cell-mediated immunity. The MLC responses of group 4 (G.I. females mated to a K.I. male) did not follow the trend shown in the other groups. Stimulation indices were very low at the beginning of the breeding season and, with one exception, increased later. The very low level of response in this breeding group may reflect the genetic disparity between the two races, since in eutherians MLC responses can be very low in xenogeneic combinations (Lafferty and Jones 1969; Wilson and Fox 1971). When G.I. females were tested against a G.I. male, the stimulation indices obtained were very much higher.

In conclusion, these studies indicate that, as in eutherians, sensitization and repeated pregnancy to the same male do not affect the reproductive efficiency of the tammar wallaby. Even though sensitization of virgin females to their prospective mate was sometimes followed by a long interval between removal of pouch young and the next birth, these females were still able to produce the same total number of young as normal females. Thus in general it appears that the tammar foetus is well protected from any deleterious immunological effects which might result from maternal sensitization to male histocompatibility antigens. However, this need not necessarily mean that the problem of foetal rejection has not played a part during marsupial evolution by preventing development of more extensive foeto–maternal contact during gestation as suggested by Moors (1974) and Lillegraven (1975).

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