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The Level and Distribution of Antibody in Syngeneic and Allogeneic Mated Pregnant Mice Pre-immunized with H-2 Alloantigens

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Summary. The antibody profile of syngeneic and allogeneic (donor) mated female mice, previously actively immunized with allogeneic spleen cells was measured during pregnancy. Marked differences were found, since syngeneic mated females showed a transient fall in antibody titre, whilst allogeneic mated females showed a strong upsurge in antibody titre, from the last week of pregnancy to well after parturition. The level of alloantibody in the serum of the syngeneic and semi-allogeneic progeny of these matings was markedly different.

Immunofluorescent studies on lymphoid cells from the semi-allogeneic progeny of immune females revealed a significant number carrying an immunoglobulin which was concluded to be alloantibody on the basis of control experiments. The differences in maternal alloantibody production profiles were attributed either to an alteration in immunoregulation of antibody synthesis in allogeneic mated females caused by absorption of antibody by the semi-allogeneic progeny *in utero*, or to secondary response to foetally-derived allogeneic antigens, or the synergistic action of both.

INTRODUCTION

It is now generally accepted that antibody can play a specific regulatory role on the immune response by combining with or dissociating from antigen to render the latter less or more immunogenic (for a full review see Uhr and Möller, 1968). Much of this knowledge has been gained from studies on the effect of passively transferred antibody. The regulatory role of serum antibody has been investigated in rabbits (Bystryn, Graf, and Uhr, 1970) and in mice (Bystryn, Schenkein and Uhr, 1971) by exchange transfusion. The protocol in both studies was to immunize with two antigens, followed by depletion of antibody to one of the antigens, and addition of antibody to the other. A rapid rebound in the titre of the depleted antibody and a simultaneous suppression in titre of the passively transferred antibody followed.

One situation in which depletion of circulating antibody might occur is the maternal circulation during pregnancy. Since IgG, but not IgM antibody reaches the foetus it might be expected that immunoregulation could compensate for the loss of antibody by exposure of immunogen and increased antibody synthesis. The results of Ralph, Nakoinz and Cohn (1972) could be interpreted along these lines, since they showed that the plaque-

forming cell response to sheep red blood cells was increased in the case of IgGl, IgG2 and IgG3 compared with IgM during pregnancy. In the present investigation, the level of antibody to alloantigens in mice was measured during pregnancies, in which the foetuses were syngeneic or semi-allogeneic with the female. The dramatic rise in alloantibody in females bearing semi-allogeneic foetuses was attributed both to the transfer of alloantibody to the foetuses *in utero* and stimulation of the mother by foetally-derived paternal alloantigens.

MATERIALS AND METHODS

Animals

Brother-sister mated BALB/c (H-2^d) and C57Bl (H-2^b) mice, bred in this laboratory were used throughout. The hybrid of these strains (BALB/c × C57Bl) F_1 is hereafter denoted CBF_1.

Immunizations

BALB/c females received weekly intraperitoneal (i.p.) injections of washed C57Bl spleen cells in 0·5 ml of Eagle's minimal essential medium (MEM, Burroughs Wellcome) the cell doses at each injection being 25, 50, 75 to 100 $\times 10^6$ cells. A final injection of 0·1 ml of C57Bl spleen cells incorporated into Freund's complete adjuvant (approximately 300–450 $\times 10^6$ cells/ml of emulsion) was given intradermally in the rear flanks, 7–14 days after the last injection. Mice were mated 2 or more weeks after the last injection, during which time they received no further injections. Alloantisera (BALB/c anti-C57Bl) were produced in the same way, and collected as described below.

Mating schedule

Mice were housed in wire cages, the time of mating being recorded by the appearance of a vaginal plug. Shortly before parturition, the females in some experiments were transferred to other hanging wire cages, and the newborn colostrum-deprived litter collected, placentae intact, in boxes under the cages.

Collection of sera

Blood from females at various stages before, during and after pregnancy was collected by puncture of the retro-orbital plexus using clean Pasteur pipettes. Usually 0·2 ml blood samples were taken which were spaced at 4–5 day (or sometimes longer) intervals. There was no evidence that this treatment *per se* resulted in changes in antibody titre. Blood from newly born colostrum-deprived mice was collected by cardiac puncture in small glass Pasteur pipettes fitted with a rubber tube and mouthpiece. Blood from each litter was pooled. Serum separated from each blood sample in the usual way was decomplemented at 56° for 30 minutes, and stored at −20° until required for use.

Cytotoxic tests

Alloantibodies in sera were identified by the following cytotoxic tests against washed C57Bl mesenteric lymph node target cells.

(1) Sera from newborn mice and their mothers at the time of parturition were analysed by the method described by Bodmer, Tripp and Bodmer (1967), with modifications. Serial two-fold dilutions of sera (2·5 μl) in MEM were added to wells in a Falcon micro-

test plate (No. 3034) with a Hamilton Microlitre (No. 702) 25 μl syringe. The borders of the plate were moistened with filter-paper strips soaked in distilled water. To each well was added 2·5 μl of target cells (1·25–2·5 $\times 10^5$/ml) labelled with fluorescein diacetate. The plate was incubated for 20 minutes in an atmosphere of 95 per cent air/5 per cent CO_2, and then 2·5 μl aliquots of fresh absorbed guinea-pig serum (see below) diluted 1/3 were added to each well. The test plates were incubated for a further 40 minutes at room temperature in 95 per cent air/5 per cent CO_2, and were then examined under a fluorescence microscope as outlined below. The results of the tests were scored as follows: positive cytotoxicity >50 per cent cells dead; negative cytotoxicity <50 per cent cells dead; endpoints were considered to be serum dilutions yielding about 50 per cent dead cells. Duplicate tests were done for each serum and endpoints never varied beyond one dilution in either direction. Normal BALB/c serum controls were carried out in parallel.

(2) Cytotoxic titrations of pregnant female alloantibody were carried out essentially by the method of Boyse, Old and Chouroulinkov (1964). The tests were performed in 5 $\times$ 50-mm glass tubes using serial two-fold dilutions of sera (25 μl) and equal volumes of target cells (5 $\times 10^5$/ml) and guinea-pig serum diluted 1/3 in MEM. The tubes were incubated for 1 hour at 37° in 95 per cent air/5 per cent CO_2, and then centrifuged lightly to sediment the cells. The supernatants were removed, and the cells resuspended in 0·16 per cent Trypan Blue in 0·9 per cent saline. End points were taken when 50 per cent of the cells were dead.

Complement

The source of complement for the cytotoxic tests was fresh serum obtained from the blood of Hartley guinea-pigs. The serum was absorbed twice with an extensively washed liver homogenate from C57Bl mice, the ratio being 10 vol. serum: 1 vol. homogenate, at 4° for 30 minutes (Haughton and McGehee, 1968). In some cases CBF_1 liver and spleen were used. The serum was freeze dried for storage and reconstituted before use. Batches tested against lymph node target cells showed no residual toxicity.

Immunofluorescence

Membrane immunofluorescent tests were carried out according to the method of Möller (1961). Cells (5–10 $\times 10^6$/ml) from CBF_1 were incubated with alloantiserum (BALB/c anti-C57Bl) or normal serum, diluted 1/10 with phosphate buffered saline (PBS) or directly with similarly diluted (1/10) fluorescein-isothiocyanate conjugated (FITC) horse anti-mouse γ-globulin (Progressive Laboratories Inc. Baltimore, U.S.A.). Cells pretreated with alloantisera or normal sera were then treated with FITC-antiserum. In some cases an unconjugated horse anti-mouse γ-globulin was used to block the staining with FITC-antiserum. Incubations were carried out in 0·1 ml vol. at room temperature for 15 minutes. Washing was repeated three times, with at least 1 ml of MEM at each wash, followed by centrifugation at 800 rev/min (MSE Minor Centrifuge) and removal of the supernatant. Cells were resuspended in 50 : 50 glycerol : saline and examined under a Wild M20 microscope equipped with an HB200 mercury light source, KG1, heat-absorbing, UG1 fluorescence-exciting and BG38 red-absorbing filters. The cells were examined for specific staining by the criteria outlined by Möller (1961).

RESULTS

ALLOANTIBODY LEVELS IN PREGNANT MICE

The anti-C57Bl alloantibody profiles during pregnancy are shown for three syngeneic

and three allogeneic mated BALB/c females in Fig. 1. The profiles have been corrected for the time of parturition which is designated day 0. The titre in the syngeneic mated females (Fig. 1a) fell between mid-term and the end of pregnancy (i.e. day-10 and day-0), the fall being two to three doubling dilutions, and was followed by a post-partum rise. The titres of the allogeneic mated females are markedly different (Fig. 1b). Two out of three females studied showed a transient fall in titre on the sixth and seventh days before parturition, whilst all showed a subsequent rise in titre during latter days of pregnancy. The rise in titre during this phase of pregnancy was three to five doubling dilutions, and in all three cases the rise was perpetuated in the post-partum period.

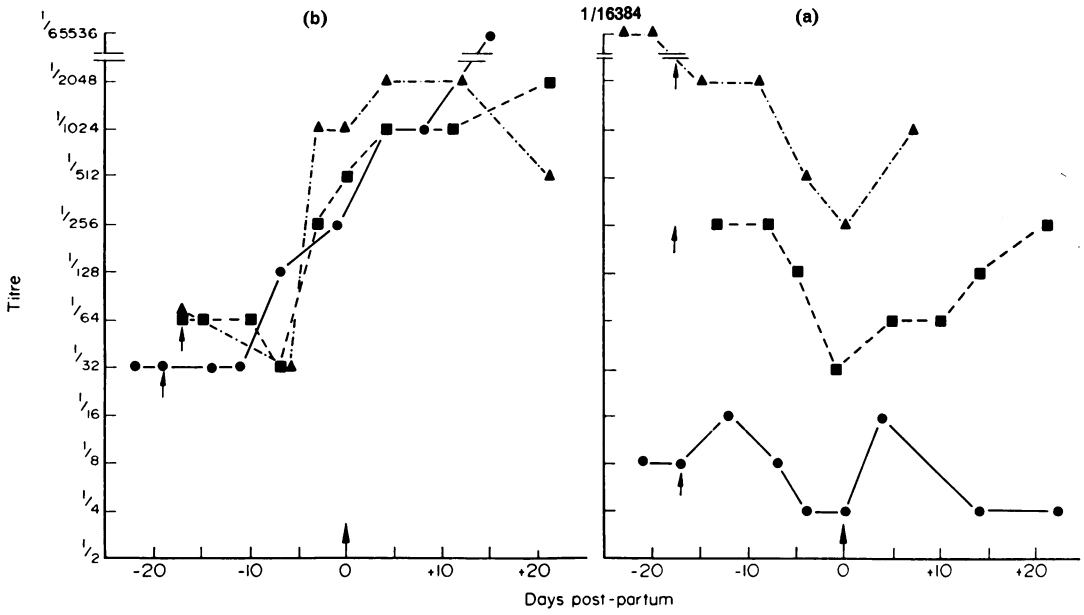

FIG. 1. Cytotoxic anti-C57Bl alloantibody profiles of BALB/c females during pregnancy, (a) three females mated to BALB/c males, (b) three females mated to C57Bl males. Parturition, indicated by broad arrow, is day 0. Date of (probable) coition indicated by fine arrow.

EFFECT OF PREIMMUNIZATION AGAINST PATERNAL ALLOANTIGENS ON LITTER SIZE

The numbers of young born to immune and non-immune syngeneic and allogeneic mated BALB/c females are shown in Table 1. All of the litters were collected in boxes under hanging wire cages as described in the Materials and Methods, so that they were unaffected by cannibalism by the mothers. The average total litter sizes are similar in the syngeneic and allogeneic crosses, with no significant differences between immune and non-immune females. There were invariably still-births in the litters of both syngeneic and allogeneic mated females. The cause of death could, in most cases, be directly attributed to the lack of maternal attention, since the litters were separated automatically at parturition. Complications thus arose because of the failure of the mothers to remove membranes, placentae and clean the young of clotted fluid. There was no significant difference in the numbers of dead progeny in the litters depicted in Table 1.

TABLE 1

THE NUMBER OF PROGENY OF NORMAL AND ALLOGENEIC SPLEEN CELL PREIMMUNIZED BALB/C FEMALES MATED TO SYNGENEIC (BALB/c) OR ALLOGENEIC (C57Bl) MALES

Type of mating	Pretreatment of females	No. of litters	Mean total litter size at parturition ±1 SE	*P**	Mean stillborn at parturition ±1 SE†	*P**
Syngeneic	None	4	6·7±1·1	>0·10	0·5±0·2	>0·10
Syngeneic	Anti-C57Bl‡	11	7·2±0·6		0·8±0·2	
Allogeneic	None	8	7·1±1·1	>0·10	1·1±0·5	>0·10
Allogeneic	Anti-C57Bl	15	7·6±0·7		1·5±0·4	

* *P* values for Student's *t*-tests comparing immune and non-immune matings.
† See text for further details of stillborn progeny.
‡ Females preimmunized with injections of C57Bl spleen.

TRANSMISSION OF MATERNAL ALLOANTIBODY TO PROGENY

The level of circulating maternally-derived anti-C57Bl alloantibody in the pooled serum from the progeny of some of the syngeneic and all of the allogeneic crosses shown in Table 1 is depicted in Table 2. The titres of the maternal and progeny sera are shown. To compensate for differences between maternal titres, the progeny titres are expressed as fractions of their respective maternal serum titres, the materno-foetal fraction (MFF). In the case of the syngeneic matings, a total of eleven immunized females and their progeny were tested at parturition. In seven out of eleven cases the maternal and progeny sera had a titre $<1/2$ and are not shown in Table 2. Of the remaining four females which had positive titres and which are shown in Table 2, the progeny titres were also positive and slightly less than the maternal titres. The average MFF was 1/2. The semi-allogeneic progeny titres could be divided into two groups, those which were positive, and those which were negative (i.e. $<1/2$). In all instances, the females had positive cytotoxic titres. The mean MFF for the positive progeny group was 1/21, considerably less than for the syngeneic group. The MFF for the negative progeny group is taken as zero since no progeny titre was detected at 1/2. Thus whilst seven out of eleven females in the syngeneic group had negative titres, all fifteen of the females in the semi-allogeneic group (i.e. mated to C57Bl males) had positive cytotoxic titres. Of the few females of the syngeneic group with positive titres, the progeny also had positive titres, whilst in the semi-allogeneic group, eight out of fifteen of the progeny sera tested were positive.

DEMONSTRATION OF MATERNALLY DERIVED ALLOANTIBODY ON THE CELLS OF SEMI-ALLOGENEIC PROGENY

To find out whether the reduced titres of CBF_1 progeny serum could have been caused by absorption of alloantibody by foetal tissues, immunofluorescent studies were carried out. In Table 3 the results are depicted for a representative experiment with cells from CBF_1 progeny of immune and non-immune BALB/c females. The cells were treated directly, after extensive washing, with FITC-anti-mouse γ-globulin and the results show that specific (ring or cap-like) staining occurred in spleen (1·95 per cent) and liver (1·45 per cent) but not the thymus. No reactions were seen in cells treated with 'blocking' serum

TABLE 2

THE LEVEL OF ANTI-C57Bl ALLOANTIBODY AT PARTURITION IN THE SERA OF BALB/c FEMALES PRE-IMMUNIZED WITH C57Bl SPLEEN AND IN THE SERA OF SYNGENEIC OR SEMI-ALLOGENEIC PROGENY

Type of mating	Cytotoxic tests against C57Bl lymph node cells			
	Maternal serum titre	*Pooled progeny serum titre	MFF†	Litter size
Syngeneic‡	1/8	1/4	1/4	6
	1/16	1/8	1/2	8
	1/32	1/8	1/4	10
	1/32	1/8	1/4	10
	1/64	1/64	1	6
		Average	1/2	7·5
Allogeneic	1/64	1/8	1/8	2
	1/128	1/8	1/16	8
	1/128	1/8	1/16	7
	1/128	1/8	1/16	11
	1/256	1/2	1/128	9
	1/256	1/4	1/64	8
	1/512	1/8	1/64	11
	1/512	1/16	1/64	8
		Average	1/21	8·0
Allogeneic	1/32	0	–	10
	1/32	0	–	9
	1/64	0	–	4
	1/64	0	–	10
	1/64	0	–	8
	1/128	0	–	10
	1/512	0	–	10
		Average	–	8·7

* MFF = materno-foetal fraction, i.e. the maternal titre ÷ foetal titre, and compensates for variations in the titre of individual females.

† These are the titres of the pooled serum from each litter, excluding dead progeny. Titres of less than 1/2 are considered to be 0.

‡ Seven syngeneic mated females and their litters had titres less than 1/2 and are not shown.

(anti-mouse γ-globulin) or with cells derived from the progeny of non-immune females, except for spleen cells which showed a very low level of staining (0·23 per cent). Other experiments performed in the same way confirmed this level of staining.

In Table 4 results are depicted for spleen, liver and thymus cells from newly born colostrum-deprived CBF_1 progeny of non-immune BALB/c females, the cells having been treated *in vitro* with BALB/c anti-C57Bl alloantiserum according to the details shown.

This treatment resulted in positive staining reactions in the order spleen (8·91 per cent) >liver (4·65 per cent) >thymus (1·98 per cent), and was blocked by anti-mouse γ-globulin. Control cell suspensions treated with normal BALB/c serum did not stain significantly, though spleen cells showed a staining reaction which may have indicated absorption of normal γ-globulin from the control serum. These results suggest, though do not prove, that a maternally-derived immunoglobulin was present on the surface of cells from the newborn CBF_1 progeny of immune BALB/c females.

TABLE 3

MEMBRANE—IMMUNOFLUORESCENT REACTIONS OF CELLS FROM CBF_1 PROGENY BORN TO IMMUNE AND NON-IMMUNE BALB/C FEMALES

CBF1 cells from progeny of:		*Cells incubated sequentially with:		No. cells positive/No. counted (per cent positive) from:		
		1. Horse anti-mouse	2. FITC-Horse anti-mouse	Spleen	Liver	Thymus
Immune BALB/c	Normal	−	+	8/410 (1·95)	6/408 (1·45)	0/353 (0)
	'Blocked'	+	+	0/487 (0)	0/479 (0)	0/360 (0)
Normal BALB/c	Normal	−	+	2/864 (0·23)	0/649 (0)	0/721 (0)
	'Blocked'	+	+	0/540 (0)	0/514 (0)	0/514 (0)

* +or− = cells incubated with a reagent or with PBS respectively.

TABLE 4

MEMBRANE—IMMUNOFLUORESCENT REACTIONS OF CBF_1 CELLS TREATED WITH BALB/C ANTI-C57B1 ALLOANTISERUM

CBF1 cells from progeny of:	Regime	*Cells incubated sequentially with:			No. cells positive / No. cells counted (per cent positive) from:		
		1. BALB/c anti-C57B1 serum	2. Horse anti-mouse	3. FITC-Horse anti-mouse	Spleen	Liver	Thymus
Normal BALB/c	Normal	+	−	+	36/369 (8·91)	16/328 (4·65)	9/444 (1·98)
	'Blocked'	+	+	+	9/243 (3·57)	1/295 (0·33)	2/442 (0·45)
		1. Normal BALB/c serum	2. Horse anti-mouse	3. FITC-Horse anti-mouse			
Normal BALB/c	Normal	+	−	+	3/316 (0·9)	1/424 (0·2)	1/322 (0·3)
	'Blocked'	+	+	+	2/205 (0·9)	0/211 (0)	0/279 (0)

* + or− = cells incubated with a reagent or in PBS respectively.

DISCUSSION

The results presented here show that female mice previously immunized with H-2 incompatible tissue have markedly elevated alloantibody titres during pregnancy when mated to males of the donor strain, whilst those mated with syngeneic males conversely showed a fall in titre. There was no evidence that the litters of allogeneic mated females were aborted as a result of the anti-paternal alloantigen immunization, or that breeding was in any way less efficient. Mitchison (1953) showed that anti-paternal strain immunization was without effect on litter sizes in mice, and Currie (1969) reached the same conclusion by assessing total numbers of conceptions and percentage foetal resorptions in spleen cell immunized allogeneic mated females.

Increases in antibody titre, similar to that observed here, follow removal of antibody from the circulation by exchange transfusion, and frequently results in higher titres than initially recorded (Bystryn, Schenkein and Uhr, 1971). It is significant that the foetal mouse first obtains IgG from the mother on the eleventh day of gestation (Morphis and Gitlin, 1970), a time which coincides with the fall in the antibody titre in the syngeneic mating, and the beginning in upsurge in antibody titre in the allogeneic mated females. Although it was not the purpose of the present study to investigate antibody levels to

heterologous antigens in pregnancy, it is possible that the alloantibody profile in the syngeneic mated females behaved in a similar way. Thus, in this group there was a decrease in alloantibody titre in the latter days of pregnancy, and seven of the eleven syngeneic mated females tested at parturition showed no alloantibody titre, in spite of having been repeatedly immunized with alloantigens.

The marked upsurge in alloantibody titre in the allogeneic mated females during the latter days of pregnancy is, in the light of the results of the syngeneic matings, attributable to the H-2 incompatibility between maternal and foetal tissues. Since alloantigens are demonstrable in embryonic mouse tissues at $10\frac{1}{2}$ days of gestation (Schlesinger, 1964), there are two possible ways in which the dramatic rise could have occurred. Firstly, absorption of alloantibody by the semi-allogeneic foetuses may have caused rapid depletion of the maternal circulation, and an abolition of feedback regulation, leading to the exposure of fresh antigen and immunogenic complexes in the pregnant female. This explanation agrees with Bystryn *et al.* (1971) showing the rapid rebound in titre following exchange transfusion. A second possibility, which may account for the antigenic stimulus mentioned above, and is thus not mutually exclusive of the first explanation, is that alloantigen derived from the semi-allogeneic foetuses may have had a 'booster' effect on the maternal response. The synergistic action of these two factors both depending on the presence of the semi-allogeneic foetuses must also be taken into account.

IgM and both IgG2a and IgG2b antibodies fix complement in the mouse (Müller-Eberhard and Grey, 1967) but IgM does not traverse the placenta whereas IgG2 does (Fahey and Barth, 1965). Antibodies causing passive lysis (IgG2) are detectable in the near-term mouse foetus at $\frac{1}{4}$–$\frac{1}{8}$ of the maternal concentration (Carretti and Ovary, 1969), a result in agreement with the present value of alloantibody in syngeneic mated females. The *in vivo* absorption of alloantibody by the semi-allogeneic foetuses could account for the lower, and often negative titres, which were broadly related to the litter size and hence the antigenic mass. A more precise relationship between foetal titre and litter size was difficult to show owing to variations in maternal titre. However, the immunofluorescent studies on suspensions of hybrid cells clearly indicated with the exception of the thymus, the presence of a surface immunoglobulin which was probably maternally derived. Since the number of immunoglobulin carrying cells was very small, verification of the type of antibody by the addition of complement to suspensions of cells from the hybrid progeny of immune mothers proved inconclusive.

The lack of an *in vivo* cytotoxic effect of IgG2 alloantibody on the foetus is not fully understood. Similar findings were reported by Lanman and Herod (1965) with hybrid progeny of rabbits, immunized with paternal skin allografts. The result presumably reflects, in part, the relative inefficiency of IgG antibodies in *in vitro* cytotoxic reactions, compared with IgM (Möller, 1966) and the paucity of alloantigens on foetal compared with adult cells (Schlesinger, 1965), since the mouse is born with its whole complement system intact (Tachibana and Rosenberg, 1966). It may also be significant to foetal survival that tumour cells destroyed *in vitro* by IgG2 alloantibody and complement are enhanced *in vivo* by these antibodies (Irvin, Eustace and Fahey, 1967).

The occurrence of anti-H-2 haemagglutinins in the sera of multiparous outcrossed mice is a well-documented phenomenon (Herzenberg and Gonzales, 1962; Kaliss and Dagg, 1964). Goodlin and Herzenberg (1964) found that the natural haemagglutinins disappeared in the third week of pregnancy and reappeared 1 week post-partum. Conversely, Kaliss and Rubinstein (1968) could find no relationship between pregnancy-

induced haemagglutinins, and the stage of pregnancy and attributed the observed variations in titre to cyclical fluctuations known to occur in the immune response. These studies relied on haemagglutination tests which could not be expected to differentiate between the different Ig subclasses. The recent work of Ralph, *et al.* (1972) indicates that the number of antibody-forming cells to SRBC in pregnancy decreases for IgM, but increases for IgG1, IGg2 and IgG3. The fact that the latter is also transported rapidly to the foetus suggests that the foetus may play a regulatory role here too. The adaptive significance of a foetal immunoregulatory mechanism toward the maternal anti-paternal alloantibody response would be assured if the foetus receives, in consequence, a degree of immunological protection against maternal cellular immunity. The finding of cellular immunity to paternal alloantigens in multiparous mice (Sören, 1967), which is abrogated *in vitro* (Hellström, Hellström and Brawn, 1969) and *in vivo* (Currie, 1969) by serum borne factors has been considered as evidence of foetal enhancement probably by immunoglobulins. Undoubtedly, the anatomical separation of maternal cellular immunity from foetal tissues is crucial to successful pregnancy; the exact role of enhancement in foetal development remains to be established.

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