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Effects of placental tissue on immunological responses

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SUMMARY

Using *in vivo* assays for alloreactivity, the responsiveness of maternal lymph node cells was shown to be increased when cells were harvested from both syngeneically and allogeneically pregnant mice. In addition, foetal liver and thymus cells induced a state of delayed type hypersensitivity to alloantigens whereas trophoblastic tissue, in the form of 12 to 15 day metrial glands, did not.

The influence of mouse placenta on *in vitro* immune responses was tested using 7-day ectoplacental cones as sources of trophoblastic tissue. Ectoplacental cones increased a T cell dependent but not a T cell independent antibody response, whereas T cell dependent cytotoxicity was reduced. The findings reported in this paper suggest that murine ectoplacental cones are a particularly useful tissue with which to study both specific and non-specific immunological activities of the trophoblast in syngeneic and allogeneic pregnancies.

INTRODUCTION

Various mechanisms have been proposed to account for the survival of the foreign embryo and foetus in the immunocompetent mother (Medawar, 1953; Beer & Billingham, 1971; Beer *et al.*, 1976). In general, current investigations into the intricacies of the foetal-maternal relationship have centred on three mechanisms: (1) the influence of maternal antibodies of a special immunoglobulin class on the expression of foetal antigens and the possibility of antigen masking or antigen modulation (Palm, Heyner & Brinster, 1971; Edidin, 1972), (2) the possibility that soluble foetal antigens, perhaps complexed with maternal antibodies, blockade maternally-derived, immunologically-aggressive cell types (Hellström, Hellström & Brawn, 1969), as well as suppressing maternal immunocompetence (Plum, Thiery & Sabbe, 1978), and (3) the production, by trophoblast or maternal decidual tissue, of molecules which induce non-specific immunosuppression in the micro-environment of the placenta (Han, 1975).

The trophoblast, located at the interface between maternal tissues and the foetus (Palm *et al.*, 1971), is likely to play a crucial role in protecting the foetus from maternal immunological aggression. This may be so, regardless of which of the above mechanisms predominates during the various stages of gestation or in different species with different placentation types. In this paper, data are presented on the influence of mouse trophoblast on various humoral and cellular immune responses mediated by mouse lymphoid cells *in vitro*. Ectoplacental cones (EPC) were used as a source of trophoblastic tissue; the ectoplacental cone is a cap-like cell mass of the polar trophoblast which is readily dissected at day 7 of gestation. (Edwards, Howe & Johnson, 1975; Theiler, 1972). The results indicate that trophoblastic tissue does

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indeed inhibit some immune responses, but facilitates others. Data are also presented on the response of maternal lymph node cells to histocompatibility antigens *in vivo* and the capacity of 12 to 15 day old metrial glands to sensitize mice against such antigens.

MATERIALS AND METHODS

Mice. Specific pathogen-free inbred CBA/H/Wehi, C57Bl/6J Wehi, BALB/cAn Bradley Wehi and the appropriate hybrids of the above strains were used. The vaginal plug dates of bred females were recorded, the day of discovery being equal to day 0 of gestation.

Cell suspensions. Cell suspensions of spleen, thymi, mesenteric lymph nodes or foetal livers were prepared by gently teasing the tissues with curved forceps through an 80 gauge stainless steel sieve in cold Eisen's balanced salt solution (EBSS). Cell suspensions of placenta (metrial glands) were prepared by aspirating minced pieces of tissue through a graded series of needles affixed to a syringe. The suspensions were gently aspirated using a Pasteur pipette and washed twice. Cell viability was determined by the eosin dye exclusion method and the cells resuspended in the appropriate medium for culture or injection into recipient mice.

Assay for graft-versus-host (GvH) reactivity of lymphoid cells. The maternal T cell-mediated reactivity to paternal antigens was measured *in vivo* by a modification of the method described by Sprent & Miller (1972). Briefly, mesenteric lymph node cells from either non-sensitized virgin CBA/H mice, primiparous CBA/H females pregnant by CBA/H males, or primiparous CBA/H females pregnant by C57Bl/6 males (taken on the 15th day of pregnancy) were injected intravenously into lethally irradiated CBA/H $\times$ C57Bl/6 F_1 hybrids which had been exposed to 850 rads whole body X-irradiation 2 to 6 hr prior to cell injection (using a Philips RT 250 machine operating at 250 KV, 15 mA, 0·2 mmCu added filter giving an HVL of 0·8 mmCu, and 50 cm source to skin distance, under conditions of maximum backscatter). Three days after irradiation, the recipient mice received 0·1 ml of 10^{-3} M 5-fluorodeoxyuridine (FUdr) by intraperitoneal injection, followed 20 min later by 2 μCi of ^{125}iododeoxyuridine (^{125}IUdr, Amersham, England, sp. act. 100 μCi/μg) (Vadas *et al.*, 1975). The spleens were excised 24 hr later and counted in a Packard autogamma scintillation spectrometer.

Assay for delayed-type hypersensitivity (DTH). The following modification of the technique described by Vadas *et al.* (1975) was used to measure the ability of foetal liver, placenta, or a combination of the two, to sensitize mice against histocompatibility antigens as assessed by the DTH reaction. Two days after receiving 200 mg/kg cyclophosphamide, CBA/H mice were injected i.p. with a cell suspension of foetal liver, placenta or both, from syngeneically or allogeneically pregnant mice. Six days later, the animals were challenged with 1×10^6 adult spleen cells in 10 μl of EBSS in the left pinna. The timing and cell concentration of this antigenic challenge were optimized in preliminary experiments. Within 4 hr following ear challenge, the mice received FUdr and ^{125}IUdr as described above; 24 hr later, the mice were killed by cervical dislocation, both pinnae were cut off at the hair-line, and placed in separate plastic tubes for gamma counting. The results were calculated as a ratio of radioactivity incorporated in the cell-injected left ear to that incorporated in the right ear (Vadas *et al.*, 1975).

Antibody responses in vitro. Seven day CBA/H or CBA/H $\times$ BALB/c F_1 conceptuses were removed from the decidual swellings under sterile conditions and the two components of the conceptus, the EPC and foetus, completely separated using cataract knives under a dissecting microscope. Following a rinse in Dulbecco's Modified Eagle's medium (DME), either two or eight of the freshly dissected EPC or foetuses were added to cultures which consisted of 5×10^6 spleen cells/ml from CBA/H donors cultured with antigen in DME in gas permeable poly-fluor-ethylene-propylene bags at pH 7·1. The culture medium contained 10% foetal calf serum (FCS, Commonwealth Serum Laboratories, Melbourne) in a total volume of 2 ml (Munder, Modolell & Wallach, 1971; Munder *et al.*, 1973). After harvesting, the number of viable cells was determined by trypan blue dye exclusion; the recovery of viable cells was between 50–70% at day 4.

Sheep red blood cells (SRBC) were used either from a pooled batch (Commonwealth Serum Laboratories, Melbourne) or from sheep kept in the Institute animal house. NIP-POL (polymerized flagellin from *Salmonella adelaide* bacteria conjugated with the hapten 4-hydroxy-3-iodo-5-nitrophenol) (Brownstone, Mitchison & Pitt-Rivers, 1966) was kindly provided by Dr M. Howard of the Walter and Eliza Hall Institute. It contained 1–2 NIP residues per monomeric subunit of POL. The number of anti-SRBC direct plaque-forming cells (PFC) was determined using the Cunningham & Szenberg (1968) modification of the Jerne plaque-forming cell assay. Cultures incubated with SRBC were assayed at day 4. Anti-NIP PFC were measured by the same technique with NIP-Fab-coated SRBC (NIP-Fab was kindly provided by Ms. J. Layton of the Institute) at day 3 of culture. The PFC count of each culture was transformed to PFC/10^6 viable recovered cells and the data expressed as arithmetic means, together with the standard error of the mean.

Mitogenicity assays. EPC and foetuses were removed as above. Either four EPC or four foetuses were added at time 0 to cultures consisting of 2×10^6 spleen or thymus cells from CBA/H mice cultured in 17 $\times$ 100 mm plastic culture tubes in 1·0 ml of RPMI 1640 supplemented with sodium bicarbonate (3·4 g/l), N-2 hydroxyethylpiperazine-N '2'-ethanesulfonic acid (HEPES, 10 mM), 1% FCS, 2-mercaptoethanol (2-ME, 5×10^{-5} M), and antibiotics (100 mg/l streptomycin sulfate and 10^5 u/l penicillin). Replicate cultures were incubated in a 10% CO_2 in air atmosphere at 37°C. DNA synthesis was measured by incorporation of ^{125}IUdr (0·1 μCi per culture) during 24–48 hr of culture. After 48 hr, the EPC or foetuses were removed from the cultures, the cells were washed three times in phosphate buffered saline (PBS), and the radioactivity determined in a gamma counter. 1·0 μg of concanavalin A (Con A, Calbiochem, San Diego, California), a concentration

known to be mitogenic for T cells (Decker, Warr & Marchalonis, 1977), was added at time 0 of incubation to either thymus or spleen cell cultures. 20 μg of *E. coli* 0111:B4 lipopolysaccharide (LPS, Difco Laboratories, Detroit, Michigan), a B cell mitogen (Decker *et al.*, 1977), were added to the spleen cell cultures.

Assay for induction of cytotoxic cells. Once EPC or foetus, either freshly dissected or pre-cultured for 1 week in 0·2 ml of DME supplemented with 10% FCS (DMEF), 2-ME, and non-essential amino acids in microtitre wells, were added to a microculture system. Both CBA/H and CBA/H × BALB/c F_1 EPC and foetuses were used. The microculture system for the induction of tumour specific immunity *in vitro* has been described by Burton, Thompson & Warner (1975). Briefly, 1×10^6 CBA/H effector spleen cells were incubated with 1×10^5 BALB/c stimulator spleen cells which were exposed first to 5000 rads of X-irradiation. The cell suspension was incubated in 0·2 ml of DMEF, supplemented as above, in microtitre wells for 1 week at 37°C in an atmosphere of 10% CO_2 in air. At the same time 100 μl of supernatant was removed from each well and replaced with 25×10^3 P815 tumour target cells labelled with ^{51}Cr in 100 μl of DMEF. P815 is an H-2^d tumour cell line, kindly supplied by Dr A. Harris of this Institute. Following a 4 hr incubation at 37°C, then 1 hr at 45°C, 100 μl of supernatant were carefully removed from each well and the radioactivity counted in a gamma counter. The results were expressed as the percentage specific lysis, with the background and maximal counts released determined according to the procedure of Burton *et al.* (1975). Each culture was performed in at least six replicates.

RESULTS

Cell-mediated immunity in vivo

Graft vs host reaction (GvH). Fig. 1 shows the GvH response of mesenteric lymph node cells from both syngeneically and allogeneically pregnant mice in irradiated F_1 mice, as well as the response of cells from virgin females. The scale on the abscissa reflects the GvH response above background, the level of incorporation of label into the spleen being expressed as a multiple of counts per minute (ct/min) above that in spleens from control F_1 hybrids injected with cell suspending medium (EBSS) alone. In all experimental groups, the response was dose-dependent, the GvH response increasing with increased numbers of injected cells. Cells from pregnant animals induced a response which was greater than that of cells from virgin mice; however, both the syngeneically and allogeneically pregnant animals showed a similar increase in reactivity.

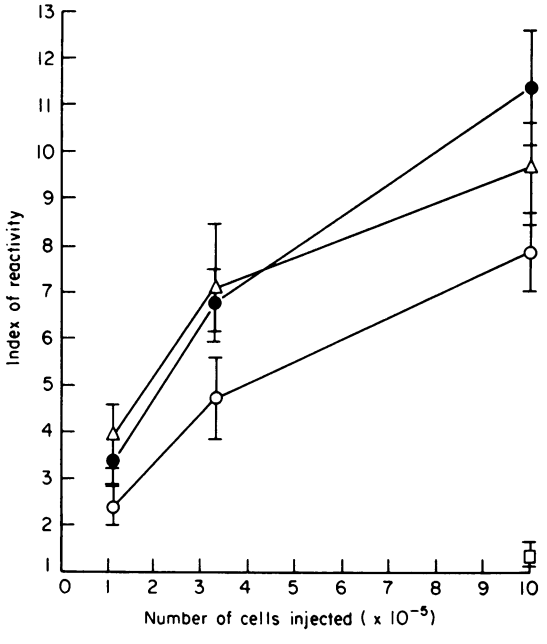

FIG. 1. GvH responses of mesenteric lymph node cells from virgin CBA/H (○——○), pregnant CBA/H mice mated C57Bl/b males (△——△) and pregnant CBA/H mice mated to CBA/H males (●——●), in irradiated and ^{125}IUdr-injected CBA/H × C57Bl/6 F_1 hybrid mice. The index of reactivity is the ratio of the radioactivity in the spleens of F_1 mice given cells to that in spleens of mice given medium alone, an index which is close to unity in F_1 mice given 1×10^6 syngeneic lymph node cells (□). Data are expressed as the arithmetic mean (±s.e.m.) obtained from five or six mice per point in two separate experiments.

Delayed-type hypersensitivity response (DTH). The ability of foetal liver or placenta to alloimmunize (as assessed by a DTH reaction) is shown in Table 1. Female CBA/H hosts pre-treated with cyclophosphamide gave a positive DTH response when injected with either 14 day CBA/H × BALB/c F_1 foetal liver or 13 day C57Bl/6 × CBA/H F_1 foetal liver and ear challenged with adult BALB/c or C57Bl/6 spleen cells, respectively. A cell suspension of one foetal liver equivalent was sufficient to produce a positive response, whilst parallel sensitization with CBA/H foetal liver did not induce a DTH response. The use of increasingly higher numbers of semi-allogeneic foetal liver cells to sensitize did not boost the response significantly, and 18 day CBA/H × BALB/c F_1 foetal thymus also induced a positive DTH (unpublished observations). Unlike the foetal tissue, the metrial glands (placenta) from the same allogeneically pregnant mice repeatedly failed to induce DTH in the CBA/H host, even if the placental tissue was derived from C57Bl/6 females mated to CBA/H males (Table 1).

TABLE 1. Ability of foetal tissues to sensitize CBA/H mice as assayed by a DTH reaction

Sensitizing tissue	Donor strain	Number of tissue equivalents	Approximate tissue weight (mg)	Number of CBA/H recipients	Left : right ear ratio of radioactivity (±s.e.m.)*
None	—	—	—	6	1·13±0·08†
14 day foetal liver	CBA/H × BALB/c F_1	2	—	6	2·20±0·19†
14 day foetal liver	CBA/H	2	—	6	1·29±0·09†
14 day placenta	CBA/H × BALB/c F_1	2	—	6	1·41±0·09†
14 day placenta	CBA/H	2	—	6	1·22±0·10†
None	—	—	—	5	1·12±0·05‡
13 day foetal liver	C57Bl/6 × CBA/H F_1	5	30	5	2·19±0·13‡
13 day placenta	C57Bl/6 × CBA/H F_1	1	30	4	1·30±0·12‡
13 day placenta	C57Bl/6 × CBA/H F_1	5	150	4	1·29±0·11‡

* Ear ratios of greater than 1·5 are considered positive in this assay (see Vadas *et al.*, 1975), the positive responses of 2·20 and 2·19 in this Table being significantly different from control values.

† Mice were challenged in the left ear with 10^6 BALB/c adult spleen cells.

‡ Mice were challenged in the left ear with 10^6 C57Bl/6 adult spleen cells.

Experiments were designed to assess the ability of placenta to inhibit *in vivo* sensitization with foetal liver cells. In each case female CBA/H mice were pre-treated with cyclophosphamide and received foetal liver cells, followed immediately by a second injection of placental tissue. Results of these experiments were variable; in all cases sensitization with foetal liver plus placenta was less than that with foetal liver injected alone. However, ear ratios of radioactivity were not usually significantly different and/or inhibition was incomplete.

Effect of EPC on antibody responses of spleen cells in vitro

The addition of CBA/H or CBA/H × BALB/c F_1 EPC to cultures of CBA/H spleen cells significantly increased (three to four times) the direct PFC response to SRBC, a T cell dependent PFC response, but the EPC had no effect on the response to NIP-POL, an antigen which induces a T cell independent direct PFC response *in vitro*. The presence of a comparable number of syngeneic or F_1 foetuses in the spleen cell cultures had a negligible effect on the response to either antigen (Table 2).

Effect of EPC on mitogenic responses of lymphoid cells in vitro

The mitogenicity of EPC was measured in order to determine whether the significant increase in anti-SRBC PFC responses noted above was due to a non-specific stimulatory effect of the EPC on spleen cells, presumably T cells. CBA/H thymus or spleen cell cultures were incubated in medium alone, or in the presence of either Con A, LPS, EPC or foetuses. Table 3 shows the results of one such experiment; these data are representative of three trials using either CBA/H or CBA/H × BALB/c F_1 EPC

TABLE 2. Influence of CBA/H and CBA/H × BALB/c F_1 7 day ectoplacental cones (EPC) and foetuses on *in vitro* PFC responses of CBA/H spleen cells to two antigens

Addition to cultures	Number of cultures (number of experiments)	Number of direct PFC per 10^6 viable recovered cells directed against: SRBC*	NIP-POL†
—	20 (8)‡	230±9	
	9 (4)		330±28
Two foetuses	18 (7)	250±11	
	7 (3)		430±92
Eight foetuses	10 (4)	240±16	
	9 (3)		490±36
Two EPC	20 (8)	670±75§	
	11 (4)		380±48
Eight EPC	9 (4)	920±158§	
	9 (3)		470±39

* Assayed at day 4 of culture, arithmetic means (±s.e.m.) indicated.

† Assayed at day 3 of culture.

‡ Results from one experiment have been omitted. In this particular experiment, the response of normal spleen cells to SRBC was unusually high (in excess of 1000 PFC per culture) and EPC did not increase this response.

§ Elevated responses in these two groups are significantly different from the response of control cultures.

TABLE 3. Effect of EPC and foetuses on ^{125}IUdr uptake by CBA/H thymus and spleen cells *in vitro*

Addition	^{125}IUdr ct/min (±s.e.m.) × 10^{-2} using: CBA/H thymus cells	CBA/H spleen cells
PBS*	9·1±1·3	17·9±2·2
Con A	51·2±10·1	110·2±3·2
LPS	n.d.	88·9±21·4
EPC	3·9±1·1	17·9±7·2
Foetuses	3·8±1·1	24·4±5·3

* PBS, phosphate buffered saline; Con A, concanavalin A; LPS, lipopolysaccharide; EPC, ectoplacental cones; and ^{125}IUdr, ^{125}iododeoxyuridine.

and foetuses where three to four replicate cultures in each group were assayed. Stimulation of thymus cells by Con A and spleen cells by LPS was at least five times above the background levels. In the presence of either EPC or foetuses from CBA or F_1 donors, no increased uptake above background was observed.

Effect of EPC on induction of cytotoxicity

The potential of EPC to alter the capacity of maternal strain T cells to respond against paternal alloantigens was measured in a two-stage *in vitro* system using ^{51}Cr release as the final assay (Burton *et al.*, 1975). The presence of freshly dissected F_1 EPC or 7 day foetuses virtually eliminated the *in vitro* generation of CBA/H anti-H-2- (BALB/c) cytotoxicity. Components of the CBA/H conceptus substantially diminished the specific lysis, but not to the levels observed with the F_1 foetal components. However, following 1 week of pre-culture of the EPC before their addition to the spleen cultures, both CBA/H and F_1 EPC significantly reduced cytotoxicity as assessed by ^{51}Cr release from P815 cells

(Table 4). In different experiments the diminution of the response was closer to 50%, although it is clear from Table 4 that the response could be blocked completely by both CBA/H and F_1 pre-cultured EPC.

TABLE 4. Effect of EPC on the generation of CBA/H anti-BALB/c cytotoxicity *in vitro*

Foetal component added to *in vitro* system*	Specific lysis of tumour target cell (P815) ±s.e.m.
None	45 ± 7
CBA/H foetus	20 ± 4
CBA/H EPC	24 ± 4
CBA/H EPC pre-cultured for 1 week	3 ± 1
CBA/H × BALB/c F_1 foetus	6 ± 2
CBA/H × BALB/c F_1 EPC	5 ± 2
CBA/H × BALB/c F_1 EPC pre-cultured for 1 week	2 ± 1

* CBA/H responder cells, irradiated BALB/c stimulator cells.

DISCUSSION

The initial objective in studying the foetal-maternal relationship was to determine whether maternal or foetal T cell dependent activities (e.g. maternal T cell dependent blocking antibodies, foetal suppressor T cells or foetal T cell dependent anti-idiotypic antibodies) play any role in the maintenance of allogeneneic pregnancies in mice. All attempts to detect such activities produced negative results. These data included a lack of effect of T cells sensitized against paternal antigens when injected at various times during gestation, on the success of allogeneic pregnancies in hypothymic nu/nu (nude) mice. Similarly, in T cell reconstituted nu/nu mice mated to allogeneic nu/+ males, litter sizes were not affected by sensitization of the mothers by injection of F_1 cells possessing paternal antigens. Moreover, no convincing evidence for specific blocking factors in maternal or foetal serum of intact mice was obtained using *in vivo* and *in vitro* T cell dependent anti-H-2 assays.

Hamilton, Hellström & van Belle (1976) reported that allogeneically pregnant mice showed an enhanced cell-mediated immune response against allogeneic embryonic cells. In the experiments reported here, it was not expected that hyper-reactivity of maternal T cell dependent proliferative responses against histocompatibility antigens would be demonstrated in both syngeneically and allogeneically primaparous pregnant mice. As shown in Fig. 1, mesenteric lymph node cells from CBA/H mice mated to either CBA/H or C57Bl/6 males, at 15 days gestation, were hyper-reactive in an *in vivo* GvH reaction using irradiated CBA × C57Bl/6 F_1 recipients. These same patterns of reactivity were seen in *in vitro* experiments reported elsewhere, although conditions could be found which demonstrated a marginally increased activity of cells from allogeneically pregnant mice (Smith *et al.*, 1978).

The trophoblast is an obvious site in which to search for evidence for protection of the foetus against aggressive maternal immunological attack (Edwards *et al.*, 1975; Bernard, 1977). Initial experiments on the trophoblast were performed using the metrial glands of allogeneically pregnant mice for the induction of DTH to histocompatibility antigens *in vivo*. Crude homogenates of semi-allogeneic (or allogeneic) metrial glands never induced sensitization, but mix experiments involving metrial glands and a sensitizing cell population, such as semi-allogeneic foetal liver or foetal thymocytes (or adult spleen cells), failed to provide clear-cut evidence for a depression of sensitization by the placenta. Using rat trophoblast, depressed *in vitro* alloreactivity of sensitized lymphocytes has been shown (M. D. Kaye and W. R. Jones, personal communication).

Studies on the effects of EPC on various immunological reactions *in vitro* have been particularly rewarding. Syngeneic or semi-allogeneic EPC in spleen cell cultures were able to increase a T cell dependent anti-SRBC antibody response, but not a T cell independent response to NIP-POL. Since

the results of *in vitro* mitogenicity studies show that EPC are not innately mitogenic for splenic T or B cells, the increase in the anti-SRBC response suggests that the EPC effect is directed at the antigen-stimulated T cell. Although we have not yet been able to detect blocking antibody in maternal serum, this stimulatory effect of EPC might reflect a role for the trophoblast in promoting synthesis of localized T cell dependent blocking antibodies.

The inhibitory effect of EPC on the generation of T cell dependent cytotoxicity *in vitro* suggests an inhibitory role of the placenta on cell-mediated immunity. This effect is also shared by foetal tissues. In these experiments, the components of the conceptus were present throughout the induction and effector stages of the two-stage cytotoxicity assay; consequently, the point of action at which inhibition takes place is unclear. However, EPC added to cultures for the first time on the day of the ^{51}Cr release assay had no effect on the cytotoxic response, nor did the addition of supernatants from cultured EPC (unpublished observations). Thus, suppression at the induction stage is suggested. Freshly dissected CBA/H $\times$ BALB/c F_1 EPC and foetuses were clearly more active at inhibition of CBA/H anti BALB/c cytotoxicity than freshly dissected CBA/H tissues. Pre-culturing of CBA/H EPC increased their inhibitory activity; this observation remains unexplained, although it is conceivable that a non-specific inhibitory effect develops later in time than an inhibitory effect of trophoblast against specific alloreactive cells. The presence of the paternal alloantigen on the F_1 conceptus may add a specific component to an otherwise non-specific effect on cytotoxic cells that occurs in the vicinity of the placenta or, if necessary, within the foetus itself. Clearly, EPC is a convenient and easily obtained tissue with which to study the molecular mechanisms of trophoblastic immunopotentiation and immunosuppression.

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