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The effect of skin allografting on the equine endometrial cup reaction

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Abstract

This research tested the hypothesis that immunological sensitization of mares by skin allografting, followed by the establishment of pregnancy using semen from the skin-graft donor, would give rise to secondary immune responses to the developing horse conceptus, resulting in an earlier demise of the fetally derived endometrial cups. Maiden mares received skin allografts from a stallion homozygous for Major Histocompatibility Complex (MHC) antigens and/or equivalent autografts and were subsequently mated to the skin-graft donor stallion during the next two breeding seasons. Mares that had been immunologically primed to the foreign MHC class I antigens of the skin graft donor stallion developed strong secondary antibody responses early in their first pregnancies, whereas autografted mares made weak primary antibody responses in their first pregnancies and strong secondary responses in their second pregnancies. In contrast, histological examination of the endometrial cups after surgical pregnancy termination at Day 60 of gestation revealed no discernible differences between allografted and autografted mares, and there were no significant differences in the concentrations and/or duration of secretion of the endometrial cup-specific hormone, equine chorionic gonadotrophin (eCG), between allografted and autografted mares, nor in either group between first and second pregnancies. The vigorous antibody response observed in the pregnant allografted mares supported the first part of our hypothesis, providing evidence of systemic immunological priming. However, there was a lack of an equivalent heightened cellular response to the endometrial cups. These findings provided strong evidence for an asymmetric immune response to the conceptus, characterized by strong humoral immunity and a dampened cellular response.

Keywords

Equine; Pregnancy; Immunology; Placenta; Trophoblast

1. Introduction

The principles of transplantation immunology [1,2] do not apply to the fetus as an allograft during pregnancy [3-6]. Half of the genetic makeup of the fetoplacental unit is derived from

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the sire and should express sufficient antigenicity to induce rejection by the dam. In species with long gestations, the semi-allogeneic fetus is carried by the dam for an interval that extends well beyond the usual time of skin allograft survival. Researchers continue to investigate new mechanisms that might underlie maternal sensitization to the fetoplacental unit and the routes of fetal evasion from maternal immune responses during pregnancy [7-10].

Because Major Histocompatibility Complex (MHC) antigens are the master molecules of tissue compatibility, decreased expression of MHC molecules on the trophoblast cells could explain how the mammalian fetus attains its privileged immunological status. In all species examined, MHC class II genes are not expressed in trophoblast cells [11]. Likewise, the majority of trophoblast cells of most species do not express MHC class I molecules [4,5,12]. However, in several species, certain trophoblast subpopulations express MHC class I antigens [4,5,13-19].

There are distinct events during early equine pregnancy that provide a reliable system for studying sensitization to paternal MHC antigens and its effects during fetal development. The chorionic girdle trophoblast cells invade the endometrium between Days 36 and 38 after ovulation where they terminally differentiate into binucleate, gonadotrophin (eCG)-secreting endometrial cup cells [20,21]. The invasive chorionic girdle cells are distinguished from trophoblast cells of other mammalian species by their expression of high levels of paternally-inherited, polymorphic MHC class I antigens [22,23]. Although these antigens become down-regulated during differentiation of the progenitor chorionic girdle cells into endometrial cup cells [24], all histoincompatible matings result in the development of high titers of cytotoxic alloantibodies directed against paternal MHC antigens within approximately 3 wk after the initial development of the endometrial cups [25,26]. These equine maternal anti-fetal MHC antibody responses are stronger, and they occur earlier in gestation, than in women or pregnant females of any other mammalian species studied [27].

In addition to the alloantibody response to the specialized invading fetal trophoblast cells, a profound cell-mediated response occurs at the interface between the fetal endometrial cup cells and the adjacent normal endometrium. A dense accumulation of maternal CD4⁺ and CD8⁺ T lymphocytes forms around each endometrial cup shortly after it develops, and the response persists and aids in the eventual degeneration of the cups and their dehiscence from the endometrium around Day 120 of gestation [28].

Because the endometrial cup cells are the sole source of the hormone, eCG, the development and eventual death of the endometrial cups during early pregnancy can be monitored by measuring maternal serum eCG concentrations. It is known that eCG helps to maintain the pregnant state by stimulating the development of progesterone-secreting secondary corpora lutea in the maternal ovaries [29,30]. Concentrations of eCG in maternal serum typically increase rapidly from Day 40 of gestation to a variable peak between Days 50 and 75, before declining in parallel with degeneration of the endometrial cups [31].

Earlier studies in rabbits and mice explored the impact of previous sensitization to foreign paternal antigens on the outcome of pregnancy. These experiments revealed that immunization of the mother with paternal skin allografts did not harm the offspring during subsequent pregnancy, nor did they alter the normal course of pregnancy [32,33]. The outcome was the same whether grafting was repeated at the time of implantation or during midpregnancy [33]. A similar study found that immunization to paternal antigens did not significantly alter placental weight, fetal weight, or litter size in the pregnant mouse [34]. However, other studies reported that maternal pre-sensitization against paternal alloantigens did significantly affect placental weight, fetal weight, and litter size [35,36]. Although these studies examined the outcome of pregnancy after presensitization to paternal antigens by skin allografting, there were no measurements of the immune response during subsequent pregnancies.

The present research tested the hypothesis that immunological sensitization of mares by skin allografting, followed by the establishment of pregnancy using semen from the skin graft donor, would result in the following outcomes: a) rapid and high-titered secondary antibody responses to the MHC class I antigens of the skin-graft donor/mating stallion; b) histological evidence of more aggressive endometrial cup reactions compared to control mares; and c) subsequent reduction in the concentrations and/or duration of secretion of the endometrial cup-specific hormone, eCG, due to the earlier demise of the endometrial cups.

2. Materials and Methods

2.1. Animals

All horses used in this study were maintained at the Baker Institute for Animal Health at Cornell University, Ithaca, NY, USA. Animal care was performed following protocols approved by Cornell's Institutional Animal Care and Use Committee. The MHC Equine Leukocyte Antigen (ELA) haplotypes of the horses were determined using a panel of alloantisera previously characterized from a series of international workshops [37] (Table 1). The skin allograft and semen donor (horse no. 0834) was a fertile stallion homozygous for the ELA-A2 haplotype.

2.2. Skin grafting

Two groups of maiden pony mares were used as skin graft recipients. In Group 1, three mares received four skin autografts each, and in Group 2, three mares received four skin autografts and four MHC-mismatched skin allografts from the single stallion homozygous for MHC class I and class II antigens. Grafts were removed from the side of the necks of donor horses and kept on ice in a sterile Petri dish containing a filter paper moistened with PBS until they were transplanted, within 1 h, to prepared sites on the side of the necks of the recipients. Donor and recipient graft sites remained bandaged until the last sutures were removed.

Group 1 mares were sedated and restrained in stocks. One side of the neck was clipped of hair and prepared aseptically before local anesthesia was achieved using 2% lidocaine at each site of skin removal. Sterile disposable biopsy punches (Miltex Instrument Company, Lake Success, NY, USA) were used to create full thickness skin autografts, ~6 mm in diameter, that were placed into an unmatched biopsy site and secured with four simple interrupted sutures of 2-0 nylon.

Group 2 mares received general anesthesia and were placed in lateral recumbency for surgery. The side of the neck was clipped and prepared aseptically. Eight recipient sites were created using disposable biopsy punches as described above and four skin autografts were placed into four unmatched recipient sites on the mares. The stallion was sedated and the side of his neck was prepared aseptically and desensitized by infiltration of local anesthetic. Skin grafts were recovered from the stallion using disposable biopsy punches and placed into the four remaining recipient sites. Each donor skin graft site on the stallion was closed with a single interrupted suture and, as before, four simple interrupted sutures of 2-0 nylon were used to secure all the skin grafts on the recipients.

2.3. Skin graft biopsies

On Days 8, 11, and 14 after grafting, both groups of recipient mares were sedated and the biopsy sites were prepared aseptically and desensitized as before. The sutures were removed from the graft sites, and punch biopsies were recovered which included recipient skin and a section of the skin graft. For Group 1 mares, one autograft site was biopsied on each recovery day, and for Group 2 mares, one autograft and one allograft site were biopsied. The remaining skin grafts were inspected carefully, and skin graft rejection was defined as the loss of the graft from the graft site on the recipient.

2.4. Establishment and termination of pregnancy

Using semen from the skin allograft donor, two consecutive pregnancies were established by artificial insemination in both groups of skin graft recipients, the first of which was established between 6 and 9 mo after the skin grafts were applied. On Day 60 of gestation, the recipient mares were anesthetized, and the conceptuses were recovered via midline hysterotomy. Samples of endometrial cups and endometrium with the allantochorion attached were collected from each mare. During the next breeding season, full sibling pregnancies were established in these recipient mares and again terminated at Day 60 for further sampling of endometrial cups and endometrium with attached allantochorion.

2.5. Tissue processing

The biopsy samples of the skin grafts and endometrial tissues were fixed immediately in 10% formalin (Fisher Scientific, Pittsburgh, PA, USA). Subsequently, sections cut from these tissues were stained with hematoxylin and eosin and examined microscopically to assess the extent of the leukocyte response. At least three to six endometrial cups per pregnancy were examined histologically.

2.6. Blood collection

Serial jugular vein blood samples were collected from the recipient mares before and after skin grafting and pregnancy. During skin grafting, blood was collected three times each week between Days 0 and 20 after grafting, and then monthly thereafter until the first pregnancy. During the first pregnancy, blood was collected three times each week between Days 0 and 90 after mating and then monthly thereafter until the second pregnancy. During the second pregnancy, blood was also collected three times each week between Days 0 and 90 after mating and then monthly thereafter for at least 2 mo. Serum was decanted and stored at -20°C . Most, but not all, serum samples were subsequently assayed for lymphocytotoxic antibody titers against donor MHC class I antigens and eCG concentrations. Heparinized peripheral blood samples were taken from the donor sire of the skin allografts. The lymphocytes were isolated from these samples and used in the lymphocyte microcytotoxicity assay described below.

2.7. Microcytotoxicity assay

A standard lymphocyte microcytotoxicity dye exclusion assay was used to measure the antibody responses to the skin grafts and subsequent pregnancies [38]. An aliquot (1 μL) of serially diluted antiserum and a 1 μL aliquot of a suspension of 3×10^6 lymphocytes/mL were incubated together for 30 min at room temperature under oil in the wells of microtiter (Terasaki) plates (Robbins Scientific, Sunnyvale, CA, USA). Five microliters of rabbit complement (PEL-FREEZ Clinical Systems, Brown Deer, WI, USA) was added and the plates were incubated for a further 1 h. The cells in each well were then stained using 2 microliters of 5% eosin dye (Fisher Scientific) before being fixed with 5 microliters of 37% formalin (Fisher Scientific), pH 7.2 to 7.4. The cytotoxic antibody titer was determined by the highest dilution of each serum that killed $\geq 80\%$ of the donor lymphocytes.

2.8. Chorionic gonadotrophin assay

An amplified enzyme-linked immunoassay [39] was used to measure serum concentrations of eCG. This assay utilized a rabbit anti-eCG serum (MSII-C) raised against a highly purified extract of eCG (MSII, 16,000 iu/mg, Dr. M.J. Stewart, Cambridge, UK). A partially purified extract of eCG (Folligon; Intervet Laboratories, Cambridge, UK) was used as standard and the limit of sensitivity of the assay was 0.5 iu eCG/mL serum.

2.9. Statistical analysis

Cytotoxic antibody titers were converted to $\log_2$ prior to statistical analysis. Unpaired Student's t-tests were used to detect differences between peak cytotoxic antibody titers generated either after skin grafting or during pregnancy between allografted and autografted mares, whereas paired Student's t-tests were used to detect differences between peak cytotoxic antibody titers generated between the first and second pregnancies of each group of mares. Statistical comparisons of peak serum eCG concentrations (iu/mL) achieved during the first and second pregnancies were performed as described above for the cytotoxic antibody titers. Differences were considered significant when $P < 0.05$.

3. Results

3.1. Equine skin allografts evoked strong cellular and humoral immune responses

Two groups of maiden pony mares received skin grafts. Group 1 received autografts only and Group 2 received MHC-mismatched allografts from a stallion homozygous for MHC class I and II antigens. Group 2 also received autografts as a means to evaluate the skin grafting technique.

Skin allografts were rejected within 14 d of grafting, whereas skin autografts survived beyond the duration of the study. A representative histological section of a skin allograft at an early stage of the rejection response (Day 8), compared to normal skin and a time-matched skin autograft is shown in Fig. 1. The leukocyte infiltrates at the interface between the skin graft and the recipient skin were evaluated histologically. On Days 8 and 11 after grafting, a large number of lymphocytes were associated with the skin allografts compared to the skin autografts. By Day 14, however, lymphocyte numbers associated with skin allografts had decreased to levels similar to those of skin autografts. On Days 8, 11, and 14 post-grafting, a large number of monocytes/macrophages was associated with both types of skin grafts when compared to normal skin. The number of neutrophils associated with the skin autografts was mildly elevated on Days 8 and 11 when compared to normal skin, but the number had decreased to background by Day 14. In contrast, few neutrophils were associated with the skin allografts on Days 8 and 11 after grafting but, by Day 14, their numbers had increased markedly. No eosinophils or plasma cells were observed in the leukocyte infiltrates associated with either type of skin graft.

Skin allografts stimulated the development of antibodies against the MHC antigens of the graft donor, but no antibodies were detected in serum samples from the autografted recipients (Fig. 2). Cytotoxic alloantibodies were detected initially at approximately Day 8 after grafting, and peak antibody titers between 1:8 and 1:32 occurred between Days 13 and 16 after grafting (Table 1). These titers declined gradually over the next 100 d.

3.2. A strong secondary antibody response occurred during pregnancy to the sire of the skin allograft

Skin graft recipient mares in both groups were artificially inseminated with semen from the skin graft donor stallion. Strong secondary antibody responses to the MHC class I antigens of the mating stallion occurred in the allografted mares during their first pregnancy (Fig. 3A). Peak antibody titers occurred between 45 and 60 d of gestation and these were higher than the peak antibody titers generated by skin allografting ($P < 0.004$; Table 1). The peak antibody responses occurred between 7 and 24 d after the invasion of the endometrium by the chorionic girdle cells.

Peak primary antibody responses were weaker in the autografted mares than in the allografted mares during the first pregnancy ($P < 0.003$; Fig. 3A). Alloantibodies first appeared in the

autografted mares between Days 42 and 60 of gestation, and peak antibody titers occurred between Days 46 and 71. These titers of 1:8 to 1:32 were similar to the peak antibody titers generated by skin allografting (Table 1).

3.3. Strong secondary antibody responses occurred during a second pregnancy of the same antigenic character

During the next breeding season, a second pregnancy was established in both groups of skin graft recipient mares using semen from the sire of the skin allografts. As a result of their prior sensitization by skin grafts and pregnancy, the allografted mares had antibody titers prior to invasion of the endometrium by chorionic girdle cells, ranging from 1:2 to 1:128 (Fig. 3B). During the secondary antibody response occurring at the time of chorionic girdle cell invasion in the second pregnancy, antibody titers reached peaks ranging from 1:256 to 1:2048 between Days 46 and 60 of gestation. The titers were not significantly different from those generated during the first pregnancy (Table 1).

As a result of sensitization by a previous pregnancy, low alloantibody titers were also present in the autografted mares prior to invasion of the endometrium by chorionic girdle cells (Fig. 3B). Strong secondary antibody responses occurred in these mares during their second consecutive pregnancies, with peak antibody titers of 1:128 to 1:2048 achieved between Days 52 and 62 of gestation (Table 1). Peak antibody titers in these autografted mares during the second pregnancy were higher than those generated during the first pregnancy ($P < 0.008$), but there were no significant differences in peak antibody titers between the allografted and autografted mares during their second pregnancy.

3.4. Skin grafting did not affect the cellular response to endometrial cups

All pregnancies were surgically terminated at Day 60 of gestation, approximately 25 d after chorionic girdle cell invasion of the endometrium. Endometrial cups were examined histologically to determine whether there were differences in the endometrial cup reaction between the allografted and autografted mares. Representative histological sections of endometrial cups recovered from mares during the first and second pregnancies after either a skin autograft or a skin allograft from the mating stallion are shown (Fig. 4). There were no discernible differences in the types or numbers of leukocytes accumulated around the endometrial cups between the allografted and autografted mares, or between the first and second pregnancies in both groups of mares. Lymphocytes were the main type of leukocyte associated with the endometrial cups in all these pregnancies.

3.5. Serum eCG profiles were similar, regardless of the source of primary or secondary sensitization

Serial serum samples collected from the mares during the first and second pregnancy were measured for eCG concentrations as another way of exploring possible differences in endometrial cup development between the allografted and autografted mares. Peak serum eCG concentrations, which ranged from 27.7 to 92.6 iu/mL in the autografted mares (coefficient of variation (CV) = 63.2%) and 13.2 to 66.0 iu/mL in the allografted mares (CV = 75.7%), occurred between Days 46 and 60 of gestation in the first pregnancy (Fig. 5A). In the second consecutive pregnancy, peak serum eCG concentrations, which occurred between Days 44 and 60 of gestation, ranged from 12.4 to 154.0 iu/mL in the autografted mares (CV = 87.9%) and 52.4 to 73.9 iu/mL in the allografted mares (CV = 17.3%; Fig. 5B). Although mean peak serum eCG concentrations were lower during the first pregnancy than the second, there were no significant differences in serum eCG profiles between the allografted and the autografted mares, nor were there any significant differences in either group of mares between the first and second pregnancies.

4. Discussion

This research tested the hypotheses that immunological sensitization of mares by skin allografting, followed by the establishment of pregnancy using semen from the skin graft donor, would result in: 1) strong secondary antibody responses to the MHC class I antigens of the skin graft donor/mating stallion; 2) histological evidence of a more aggressive endometrial cup reaction compared to control mares; and 3) subsequent reduction in the concentrations and/or duration of secretion of the endometrial cup-specific hormone, eCG, due to earlier demise of the endometrial cups. As hypothesized, prior exposure to paternal histocompatibility antigens in a non-uterine site primed for strong secondary antibody responses to paternal MHC class I antigens expressed by the chorionic girdle and early endometrial cups in the uterus during pregnancy. However, the anamnestic antibody response to the paternal MHC class I antigens during pregnancy did not alter the endometrial cup reaction, or the natural development of the endometrial cups. These findings were supported by the observation that serum eCG profiles in mares previously grafted with MHC mismatched skin from the mating stallion were equivalent to those in mares grafted with their own skin.

We used a single MHC homozygous stallion as the donor of the skin allografts and as the sire to establish the pregnancies in the grafted mares. Prior exposure to the stallion's histocompatibility antigens by skin allografting primed the mares to make strong secondary antibody responses during pregnancy. We inferred that the MHC class I antigens present on cells in adult skin were shared with those expressed by the specialized trophoblast cells of the chorionic girdle. Results from other studies supported this conclusion. Pieces of allogeneic chorionic girdle, when transplanted to non-uterine sites, sensitized the recipient mares to paternal MHC class I antigens [40]; this priming lead to strong secondary antibody responses during a subsequent pregnancy sired by a stallion carrying the same MHC type (A. P. Adams and D. F. Antczak, unpublished data). Alloantibodies raised during pregnancy immunoprecipitated MHC class I antigens from the lymphocytes of the mating stallion [41] and from chorionic girdle cells from a conceptus sired by the same stallion [23]. Furthermore, Northern blot analysis revealed that horse lymphocytes and chorionic girdle cells express similar levels of MHC class I and beta₂-microglobulin mRNA, and that all MHC class I loci transcribed in lymphocytes were also represented in chorionic girdle cells, including transcripts from both polymorphic and nonpolymorphic loci [42].

Pregnancy-induced immunization also primed for stimulation of a strong secondary antibody response to a second pregnancy of the same antigenic character. As expected, autografted mares made no antibody responses to their own skin grafts; when these autografted animals were mated to a histoincompatible sire, a primary antibody response was evoked by paternal MHC class I antigens expressed on the invasive chorionic girdle cells. A vigorous secondary antibody response occurred after the second mating, thereby supporting previous evidence of anamnestic immune responses to allogeneic conceptuses in pregnant horses, mice, and rats [26,43,44].

Regardless of the source of primary sensitization to paternal histocompatibility antigens, secondary antibody responses during subsequent pregnancy were sustained long after termination of the pregnancy and the normal lifespan of the endometrial cups. Microchimerism, or the harboring of cells from another individual at low levels, could explain the source of paternal antigen stimulation after a prior pregnancy. In humans, it is well recognized that cells traffic in both directions between the fetus and mother during pregnancy [45]. Moreover, fetal cells have been found to persist for years in the circulation of healthy women [46], and such cells may also play a role in the pathogenesis of autoimmune disease [47]. There have been no reports to date of long-term fetal-maternal microchimerism in the horse, but the migrating trophoblast cells of the chorionic girdle could be a source of such microchimerism.

Our results showed a striking disparity between the strong secondary alloantibody responses to the developing conceptus and the lack of evidence for equivalent secondary cellular immune responses associated with the endometrial cups. There were no discernible differences in the leukocyte responses around the endometrial cups in the autografted versus the allografted mares, which equates well with previous studies in which no differences were detectable in the lifespan or the cellular response associated with the endometrial cup reaction in mares carrying MHC class I compatible and incompatible pregnancies [48]. Cell recruitment by factors other than paternal MHC class I antigens could explain the reproducible migration of lymphocytes to the endometrial cups. Minor histocompatibility antigens expressed on invasive trophoblast cells may evoke a lymphocytic response [48], or chemokines secreted by the invading trophoblast cells may recruit lymphocytes from the peripheral circulation to the site of the endometrial cups [49]. Alternatively, cell recruitment to the endometrial cups may require coordinate expression of both chemokines and minor histocompatibility antigens. For example, a chemokine, known as monokine induced by interferon-gamma (Mig), has been shown to mediate optimal recruitment of T lymphocytes during the rejection response of MHC-matched/multiple minor histocompatibility antigen-disparate skin allografts [50].

Other studies have measured eCG in serum as an indirect way to monitor endometrial cup lifespan, since the endometrial cup cells are the only source of eCG. Interestingly, the results of those studies suggested an antigenic basis for eventual destruction of endometrial cups. For example, when a stallion and mare that had been born as co-twins were mated, eCG concentrations in the serum of the mare were higher and detected 50 d longer than in equivalent control mares [51]. In this case, the immune systems of the twins were tolerant of one another's histocompatibility antigens. This immunological tolerance apparently prolonged the lifespan of the endometrial cups and resultant production of eCG by either reducing or completely negating the mare's normal cell-mediated response against paternal histocompatibility antigens. These results echo the earliest observations in freemartin cattle that lead to the discovery of immunological tolerance [52,53]. By contrast, in interspecies mule pregnancy, mares mated to a donkey sire produced lower concentrations of eCG for a shorter period of gestation during their second pregnancies than during their first pregnancies to the same donkey sire, consistent with an anamnestic cellular immune response causing an earlier demise of the mule endometrial cups [54].

In the present study, there were no significant differences in the concentrations of eCG produced by the endometrial cups in mares previously grafted with MHC-mismatched skin from the mating stallion, compared to mares autografted with their own skin. Robust secondary antibody responses to the paternal MHC class I antigens did not appear to affect the migration of the chorionic girdle cells into the endometrium, or the natural process of development of the endometrial cups in these pregnancies. Similarly, there was no evidence that sensitization to paternal MHC antigens by skin grafting enhanced the maternal cell-mediated immune attack against the fetal endometrial cup cells. These findings supported previous evidence of a generalized shift away from cell-mediated immunity toward antibody-mediated immunity during normal intraspecies horse pregnancy that fails to occur during interspecies mule pregnancy [55]. After *in vitro* stimulation with irradiated allogeneic (paternal) stimulator lymphocytes, peripheral blood lymphocytes from mares carrying horse conceptuses exhibited profound decreases in MHC class I specific cytotoxic T lymphocyte (CTL) activity when compared to non-pregnant controls; in contrast, mares carrying mule conceptuses did not show this pregnancy-associated decrease in CTL activity [55].

The transient state of hyporesponsiveness to paternal alloantigens during horse pregnancy may reflect the development of maternal T cell tolerance to specific paternal alloantigens. Studies in mice with transgenic T cell receptors (TCRs) provided evidence that pregnancy disrupted maternal CTL responses to paternal histocompatibility antigens. Transgenic TCRs specific for

the MHC class I antigen H-2K^b were transiently down-regulated in mice carrying H-2K^b-positive conceptuses; H-2K^b-positive tumor grafts were accepted during these allogeneic pregnancies [56]. In another study, mice expressing a TCR transgene specific for the male minor histocompatibility antigen H-Y were used to follow the fate of maternal T cells reactive to a paternal alloantigen; some H-Y-specific T cells were clonally deleted, whereas others were unresponsive to antigenic stimulation without showing down regulation of their receptors [57]. Most recently, regulatory T cells (Tregs) have been associated with modulation of maternal immune responses to the fetus in mice and humans [6,58-61]. Collectively, these studies provided evidence for multiple mechanisms of antigen-specific and -nonspecific T cell tolerance that protected the fetal allograft from maternal immune rejection.

In summary, the findings of this study revealed a strong mechanism of fetal protection during early equine pregnancy, in that prior sensitization to paternal antigens by skin allografting did not compromise the migration of the highly immunogenic trophoblast cells of the chorionic girdle into the endometrium nor the development and normal lifespan of the endometrial cups.

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Fig. 1.

Histological evaluation of the cellular response to equine skin allografts. Hematoxylin and eosin staining was performed on biopsies of formalin-fixed samples of skin autografts and allografts taken on Day 8 after grafting. (A) Low magnification view of normal skin. (B) Higher magnification view of the boxed area in (A). (C) Low magnification view of a skin autograft on Day 8 after grafting. (D) Higher magnification view of the boxed area in (C). Note the scarcity of mononuclear cells (arrow) at the interface between the viable skin autograft (au) and recipient skin (r). (E) Low magnification view of a skin allograft on Day 8 after grafting. (F) Higher magnification view of the boxed area in (E). Note the large number of mononuclear cells (arrow) at the interface between the dying skin allograft (al) and recipient skin (r). Bar = 400 micrometers (A, C, and E) and 100 micrometers (B, D, and F).

Fig. 2.

Time course of cytotoxic anti-paternal antibody to donor antigens in horses receiving skin allografts. Skin allografts (solid symbols) elicited strong primary cytotoxic antibody responses against the MHC antigens of the skin graft donor, but no cytotoxic antibodies were detected in sera from autografted recipients (open symbols).

Fig. 3.

Anamnestic cytotoxic anti-paternal antibody responses in mares following skin allografting. Allografted mares produced strong secondary cytotoxic antibody responses when bred to the skin graft donor sire (A, solid symbols). In contrast, autografted mares produced weak primary antibody responses when bred to the same sire (A, open symbols). During the second pregnancy to the skin graft donor sire, allografted mares made strong tertiary antibody responses (B, solid symbols), whereas autografted mares made strong secondary antibody responses (B, open symbols).

Fig. 4. Histology of the cellular response to endometrial cups in mares during first and second pregnancy following either a skin autograft or a skin allograft from the mating stallion. Hematoxylin and eosin staining was performed on formalin-fixed endometrial cup tissues from Day 60 of pregnancy. Cellular responses to endometrial cups were compared between the first (A, C) and second (B, D) pregnancies of autografted (A, B) or allografted (C, D) mares. Note the large number of mononuclear cells (arrow) at the interface between the endometrial cup (ec) and the normal endometrium (em). Bar = 200 micrometers (A-D) and 500 micrometers (insets of A-D).

Fig. 5.

Serum eCG concentrations measured in mares during their first and second consecutive pregnancies following either a skin autograft or a skin allograft from the mating stallion. Serial serum samples were collected during the first (A) and second (B) pregnancies and analyzed for eCG concentrations using an amplified enzyme-linked immunoassay [39]. There were no significant differences in peak serum eCG concentrations between allografted (solid symbols) and autografted (open symbols) animals during first (A) and second (B) pregnancies, nor in either group between first (A) and second (B) pregnancies.

Peak cytotoxic antibody titers in mares sensitized to MHC antigens by skin allografting and/or pregnancy

Table 1

Group	Recipient ^a	ELA (MHC) type ^b	Skin graft	Peak antibody titer following: First pregnancy	Second pregnancy
1 (autografts)	A	A6/W16	0	1:32	1:1024
	B	A6/A7	0	1:16	1:2048
	C	?/?	0	1:8	1:128
2 (autografts + allografts)	D	A1/A6	1:32	1:256	1:256
	E	A2/? ^c	1:8	1:1024	1:2048
	F	A9/A19	1:32	1:2048	1:1024

^aRecipient mares A, B, C, D, E, and F corresponded to horse identification nos. 2469, 2470, 2516, 2471, 2474, and 2488, respectively.

^bThe MHC class I phenotype of each pony mare was determined by serological typing of lymphocytes against a panel of alloantisera established by the Fifth International Workshop on Lymphocyte Alloantigens of the Horse [37]. ? indicates that the animal carries an allele or alleles for which no antisera are available.

^cRecipient mare E shared one polymorphic MHC class I locus with the skin graft donor and mating stallion (horse no. 0834), but also carried a distinct MHC class I allele at a second locus, and a disparate MHC class II region [41].