

Ectopic Trophoblast Allografts in the Horse Resist Destruction by Secondary Immune Responses¹

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ABSTRACT

Invasive trophoblast from Day 34 horse conceptuses survives in extrauterine sites in allogeneic recipients that are immunologically naive to donor major histocompatibility complex class I antigens. The ectopic trophoblast retains its in utero characteristics, including similar lifespan, physiologic effect of its secreted product (equine chorionic gonadotropin) upon the recipient's ovaries, and induction of host immune responses. Immunologic memory has not been considered previously in this experimental system. We hypothesized that primary exposure to ectopic trophoblast would affect the recipient's immune status such that the survival time of subsequent transplants would be altered. Secondary transplant lifespans could be shortened by destructive primary responses, as has been observed in ectopic trophoblast studies in rodents, or lengthened, as occurs when male skin grafts follow multiple syngeneic pregnancies in mice. Eight mares received two closely spaced trophoblast transplants. Both grafts for each recipient were obtained from conceptuses sired by the same stallion to provide consistency in histocompatibility antigen exposure. Donor stallions were major histocompatibility complex class I homozygotes. Cytotoxic antibody production was tracked to monitor recipients' immune responses to the transplants. Detection of serum equine chorionic gonadotropin was used as a proxy for transplant lifespan. There was no significant difference between the distributions of primary and secondary transplant lifespans, despite evidence of immunologic memory. These data demonstrate that secondary ectopic trophoblast transplants in horses do not experience earlier destruction or prolonged survival following immune priming of recipients. Mechanisms responsible for the eventual demise of the transplants remain unperturbed by secondary immune responses or chronic antigenic exposure.

equids, immunology, reproductive immunology, transplantation, trophoblast

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INTRODUCTION

The complexity and redundancy of pathways that protect tissues of fetal origin from maternal immune destruction [1–3] pose a challenge to the elucidation of individual mechanisms. The phenomenon of immunologic memory may further complicate efforts to understand these events by modifying the maternal immune environment such that the response to a second pregnancy differs from that of the first. Historically, fetal survival has been considered in the context of classical transplant immunology, with the maternal immune system assumed to be a threat to the fetus because of fetal expression of foreign (paternal) major histocompatibility complex class I (MHC-I) molecules [4]. A central tenant of classical transplant immunology is that MHC-I molecules expressed on virtually all somatic cells are the trigger for the rejection of conventional tissue transplants. Furthermore, an incompatible primary transplant induces a memory response that results in more rapid rejection of an identical secondary transplant [5].

This paradigm is called into question at the maternal-fetal interface, which is increasingly viewed as a distinct entity with unique, defining characteristics that do not conform to the immunologic principles governing classical transplants. These characteristics can vary across species based on placental architecture, and the degree of invasiveness and antigenicity of the trophoblast [6]. In some species MHC-I expression by trophoblast deviates from that of typical somatic cells in ways that mitigate the maternal response. Although in and of itself this is a protective mechanism, it makes quantification and therefore study of the maternal immune response more difficult. Human trophoblast falls into this category [7, 8]. Extravillous trophoblast expresses minimally polymorphic, nonclassical human leukocyte antigen G (HLA-G), along with HLA-E and classical HLA-C. The molecular structure of HLA-G may render it unable to present antigen, providing a measure of protection to the fetus [9]. Villous cytotrophoblast does not express cell surface histocompatibility antigens. Mouse trophoblast expresses classical H2 molecules on giant cells of the ectoplacental cone, but their proposed role is to bind uterine natural killer cells involved in decidual artery remodeling [10]. By contrast, the invasive trophoblast cells of the equine chorionic girdle express high levels of both maternal and paternal classical MHC-I molecules, with paternal MHC-I inducing a strong, measurable maternal immune response in MHC-I-mismatched pregnancies [11–13]. This exposure of fetal foreign antigens to the maternal immune system seems counterintuitive, and provides an intriguing biologic system for the study of maternal tolerance.

Previous studies in our laboratory have used ectopic trophoblast transplantation in the horse to investigate the maternal immune response to trophoblast isolated from the hormonal milieu of pregnancy. This system has several

advantages arising from intrinsic characteristics of the invasive trophoblast. First, the chorionic girdle tissue has a defined lifespan of about 80 days, during which time it produces equine chorionic gonadotropin (eCG) measurable in the dam's serum [14]. Second, MHC-I molecules expressed by the chorionic girdle induce a humoral immune response upon trophoblast invasion in MHC-I-incompatible pregnancies [15], as well as rapid local infiltration by leukocytes in all pregnancies [16]. When chorionic girdle is transplanted subcutaneously in nonpregnant recipient mares, the tissue retains these in utero characteristics, with eCG measurable in the recipient's serum for a median of 75 days, and cellular and humoral responses to the trophoblast occurring as in normal equine pregnancy [17, 18]. This enables serum-based, noninvasive monitoring of the transplant's survival time and the recipient's immune recognition of the transplant. Serum eCG is used as a proxy for transplant lifespan, and cytotoxic antibodies as confirmation of the recipient's immune recognition of the transplant.

The effect of immunologic memory on the survival of ectopic horse trophoblast has not been studied. We hypothesize that the recipient's immunologic response to a primary transplant would result in a change of lifespan for second and subsequent transplants. Lifespan could be decreased by accelerated immune destruction from a memory response, as observed in serial ectopic transplant studies in mice [19], or become longer, as observed in male skin grafting experiments in mice in which recipients' immune systems were primed by multiple syngeneic pregnancies [20]. The latter could occur as a result of regulatory T-lymphocyte memory [21–23] or other mechanisms of induced tolerance, such as T-cell exhaustion resulting from prolonged antigen exposure [24].

MATERIALS AND METHODS

Animals

Horses used in this study were part of the Cornell University Equine Genetics Center herd. Recipient and donor mares used for the trophoblast transplants were reproductively mature animals of various ages and breeds, and of known Equine Leukocyte Antigen (ELA) haplotype. The donor stallions were Thoroughbreds homozygous for the ELA-A2 or ELA-A3 haplotype. Conceptuses sired by the same stallion were used for both primary and secondary transplants in each recipient. This ensured that invasive trophoblast used for the transplants carried the same MHC-I antigens to provide both the primary and secondary immune challenge, because consistency of donor mare MHC-I was not feasible. Animal care and experimental design were in compliance with protocols approved by the Cornell University Institutional Animal Care and Use Committee, and with the Guide for the Care and Use of Agricultural Animals in Research and Teaching.

One or both of two methodologies was used to determine ELA haplotypes of the horses used in this study: a serological assay based on a panel of alloantisera validated in international workshops [25], and a molecular technique based on polymorphic microsatellite loci on ECA 20 within the MHC region [26]. Stallions and recipient and donor mares that were longstanding members of the Cornell research herd were MHC typed by both serology and microsatellites. Mares acquired from other sources specifically for this study were typed only by microsatellites. This provided sufficient information to determine compatibility with donor sires and dams.

Breeding, Conceptus Recovery, and Transplantation Technique

Donor mares were impregnated via artificial insemination with fresh semen from a donor stallion. Conceptuses were recovered at Day 34 by noninvasive uterine lavage using an aseptic technique as previously described [27]. Transplants were performed in a manner consistent with a method previously described [18]. Briefly, after recovery the entire chorionic girdle was dissected, minced, loaded into a syringe with 1 ml of phosphate-buffered saline and penicillin/streptomycin, and injected aseptically into the vulvar mucosa of the recipient. Serial transplants were placed on alternating sides of the vulva.

Sampling

Blood samples were drawn via jugular venipuncture of recipient mares into serum tubes two to three times per week until the serum eCG concentration fell below pretransplantation (nonpregnant) levels. We have interpreted this return to baseline as signifying the end of the transplant's lifespan. Blood was allowed to clot, tubes were centrifuged, and serum was stored at -20°C until use. Blood from the donor stallions was drawn as needed into heparinized tubes and processed immediately for use in the cytotoxicity assays.

eCG and Progesterone Measurements

Detection of eCG above baseline values in a recipient's serum was considered evidence of living trophoblast, and therefore serum eCG concentration was used as a proxy for transplant lifespan. Serum progesterone concentration was tracked as an indicator of the status of the recipient's reproductive system. Both serum eCG and progesterone were quantified using commercially available ELISA kits (PMSG and Progesterone ELISAs, DRG International, Springfield, NJ) according to manufacturer's instructions. Plates were read on a Multiskan Ex, and data were analyzed with Ascent 2.6 software (ThermoFisher Scientific, Waltham, MA).

Lymphocyte Microcytotoxicity Assay

Serum cytotoxic antibodies generated by a recipient in response to an MHC-I-mismatched transplant were used as a read-out for the recipient's immune recognition of the transplant. The presence of antibodies also confirmed technical success of the transplant, and served as a measure of comparison for primary versus secondary immune responses. The lymphocyte microcytotoxicity assay was used as previously described [28] to detect and quantify these alloantibodies. Briefly, serum from recipients was plated in microtiter plates (Robbins Scientific, Mountain View, CA) in two-fold dilutions. Serum was incubated with lymphocytes isolated from heparinized venous blood from the donor stallion and rabbit complement (Pel-Freez, Lot no. PF29521, Brown Deer, WI). Eosin dye was added and the wells were fixed with formalin. Wells were scored as percent cell killing indicated by dye exclusion. Wells showing greater than 50% killing were considered positive. Titters were called as the highest dilution with a positive result. Each sample was tested at least twice.

Transplant End Points

The presence of eCG in the recipients' sera was considered an indicator of live and functioning trophoblast. The end point of each transplant was determined based on a previously described approach used for single ectopic trophoblast transplant studies [18], with minor modification to accommodate the timing of pregnancies from which to obtain trophoblast tissue for the secondary transplants. Transplant lifespans were generally considered terminated as eCG approached a concentration of at least twice baseline with a drop of less than 50% in the previous 6 days.

Graphing and Statistical Analysis

Data were analyzed and graphs were created using Prism 6 for Mac OS X (GraphPad Software, La Jolla, CA). Our sample-size justification confirmed 90% power for detecting a difference of 14 days between mean survival times in primary and secondary transplants with eight mares. Nonparametric testing was performed using the Wilcoxon signed-rank test. Statistical analysis was performed with Statistix10 2013 (Analytical Software, Tallahassee, FL).

RESULTS

Animals and Transplants

Twenty-one ectopic trophoblast transplants were monitored for the duration of their lifespans in eight recipient mares. All transplantations took place during two consecutive equine breeding seasons, "season 1" and "season 2" (Fig. 1). In our core experiment (Fig. 1a), we performed paired serial trophoblast transplantations in eight recipient mares. Each recipient received a primary transplant followed by a secondary transplant as serum eCG concentrations produced by the primary transplant approached pretransplantation levels. Lifespan data from these eight paired transplants were subjected to

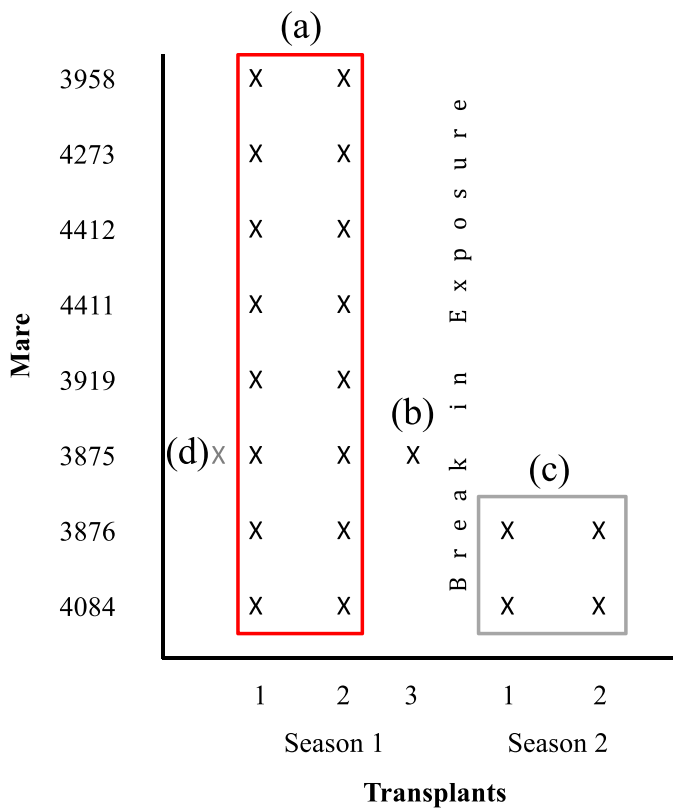


FIG. 1. Schematic diagram depicting the ectopic invasive horse trophoblast transplants performed in recipient mares. Twenty-one ectopic trophoblast transplants (each indicated by a black X) were performed and monitored for the duration of the transplants' lifespans in eight recipient mares. All eight mares first received paired serial transplants (a). At the conclusion of the paired transplant experiment, an additional transplant was performed and monitored in mare 3875 (b). The following breeding season, mares 3876 and 4084 received another set of paired transplants, shown within the gray box (c). In recipient 3875, two chorionic girdles of identical origins were overlapped at the start of the experiment to increase the "dose" of antigen (d). This transplant (indicated by the gray X) was not included in subsequent calculations. Statistical analysis was performed on the paired transplants depicted within the red box.

statistical analysis to test our stated hypothesis. At the conclusion of the paired serial transplant study, recipient 3875 received a third trophoblast transplant (Fig. 1b). The two-transplant series was repeated in the next breeding season using only mares 3876 and 4084 following a break in exposure, referred to as "season 2" (Fig. 1c). In recipient 3875, the initial exposure (Fig. 1d) was made up of two chorionic girdles from the identical donor sire and dam mating combination transplanted in rapid succession to prolong contact with antigen. The secondary transplant was then performed as described above, when eCG approached baseline.

In previous studies of single ectopic trophoblast transplants, lifespan appeared to be unaffected by the MHC-I compatibility of the recipient with the donor sire and dam [18]. In the current study we used primarily (19 of 21 transplants) transplants that were MHC-I incompatible between the homozygous donor sire and the recipient so that the primary and secondary (memory) responses could be quantified (Table 1) as evidence for memory. Only recipient 3919 received transplants from an MHC-I-compatible sire, with one being from an incompatible donor dam and one from a partially compatible donor dam. Seven of the eight recipients were maidens. Recipient 4273 was an Icelandic mare that had previously been bred to an

Icelandic stallion and produced a healthy foal. She had no circulating antibodies to the stallions in our study, and was therefore accepted as a recipient.

There Was No Significant Difference in the Distributions of Lifespan of Primary and Secondary Transplants

In the core paired serial transplant experiment (Fig. 1a), all primary trophoblast transplants produced detectable eCG in the serum of the recipient mares. Primary transplant lifespans determined from the resultant eCG curves ranged from 47 to 85 days (Fig. 2). All recipient mares were naive to the MHC-I antigens of the donor sire at the start of the experiment. The seven recipients receiving transplants from incompatible donor sires developed cytotoxic antibodies to the donor sire MHC-I. Recipient mare 3919 developed cytotoxic antibodies to the incompatible donor mare following the primary transplant (Fig. 3). The lifespans of these primary transplants were consistent with those previously reported for single trophoblast transplants [18].

Secondary transplants were performed between 0 and 37 days from the end point of the primary transplant. All secondary transplants produced eCG that could be detected in the recipient's serum. Transplant lifespans were calculated from the eCG curves (Fig. 2), and ranged from 24 to 228 days. Five of the eight recipient mares still had circulating cytotoxic antibodies from the primary transplants at the time the secondary transplants were performed. All seven incompatible secondary transplants induced increases in cytotoxic antibody titers against the MHC-I antigens of the donor sire. Six of the seven rises in titer were detected earlier after the secondary transplants than after the primary transplants, consistent with an anamnestic response (Fig. 4). Recipient mare 3919, as expected, did not produce cytotoxic antibodies to the MHC-I of the compatible donors (Fig. 3).

There was no significant difference between the distributions of the lifespans of primary (median, 66 days) and secondary (median, 88 days) transplants (Fig. 5) performed in the eight recipient mares during season 1 ($P = 0.0781$). The actual differences observed in days of survival (with a positive difference meaning that the primed-phase survival was longer than the naive-phase survival) were: -23, -4, 1, 23, 24, 25, 26, 82, and 143.

To summarize, the survival times of primary ectopic trophoblast transplants placed into naive recipient mares did not differ significantly from the survival times of secondary ectopic trophoblast transplants placed into recipients with primed immune systems.

Paired Transplants Performed During a Subsequent Breeding Season in Two of the Recipients Had Comparable Lifespans to the First Set of Transplants

After a winter seasonal break in exposure, mares 3876 and 4084 received another set of two closely spaced MHC-I-incompatible transplants (Table 2). This was done to observe the possible effects of a rest period without ongoing antigen exposure. The primary and secondary trophoblast transplants produced eCG detectable in the recipients' serum, although at notably lower concentrations than those produced by the paired transplants in these mares in the first breeding season (Fig. 6, A and B). Both recipients still had circulating anti-donor antibodies present at the time of the primary transplant in the second breeding season, and showed increases in titer following the secondary transplants (Fig. 6, C and D). Calculated lifespans ranged from 52 to 90 days, with median

TABLE 1. Season 1 paired serial trophoblast transplant recipient and donor mares and stallions.^a

Parameter	Recipient ^b	Donor stallion	Transplant 1 donor mare ^b	Transplant 2 donor mare ^b
Horse ID #	3958 ^c	3474	3725	4065
Serological ELA haplotype(s)	A2/A10	A3/A3	A2/?	A19/?
MHC microsatellite haplotype(s)	A2/novel	A3b/A3c	novel/novel	A9a/A19
Year of birth	2004	1999	2003	2000
Horse ID #	3876 ^d	3474	3640	4069
Serological ELA haplotype(s)	A2/?	A3/A3	A3/?	A1/?
MHC microsatellite haplotype(s)	A2/novel	A3b/A3c	A3b/novel	novel/novel
Year of birth	2006	1999	1998	2002
Horse ID #	4084 ^e	3474	3957	3957
Serological ELA haplotype(s)	A5/A9	A3/A3	A1/A3	A1/A3
MHC microsatellite haplotype(s)	novel/novel	A3b/A3c	005/novel	005/novel
Year of birth	2006	1999	2003	2003
Horse ID #	3919 ^f	3475	4266	3876
Serological ELA haplotype(s)	A2/A2	A2/A2	ND	A2/?
MHC microsatellite haplotype(s)	A2/A2	A2/A2	Ice02/Ice04	A2/novel
Year of birth	2007	1999	2007	2006
Horse ID #	4273 ^g	3475	4231	3880
Serological ELA haplotype(s)	ND	A2/A2	A3/A3	A3/A3
MHC microsatellite haplotype(s)	Ice17/Ice18	A2/A2	A3b/A3c	A3a/A3b
Year of birth	2004	1999	2011	2006
Horse ID #	4412 ^h	3475	3601	4265
Serological ELA haplotype(s)	ND	A2/A2	A3/A3	ND
MHC microsatellite haplotype(s)	Not A2 ^k	A2/A2	A3a/A3b	Ice05/Ice06
Year of birth	2006	1999	2001	2005
Horse ID #	4411 ⁱ	3475	3908	3880
Serological ELA haplotype(s)	ND	A2/A2	A2/A2	A3/A3
MHC microsatellite haplotype(s)	A5a/novel	A2/A2	A2/A2	A3a/A3b
Year of birth	2004	1999	2007	2006
Horse ID #	3875 ^j	3474	3640	3640
Serological ELA haplotype(s)	A2/?	A3/A3	A3/?	A3/?
MHC microsatellite haplotype(s)	A2/novel	A3b/A3c	A3b/novel	A3b/novel
Year of birth	2006	1999	1998	1998

^aELA (MHC) Haplotypes: the two donor stallions were bred for MHC class I homozygosity at the ELA-A2 and ELA-A3 haplotypes. Other serological (ELA) designations are shown when known. ? indicates MHC haplotype for which no identifying alloantisera have been developed. Some microsatellite haplotypes correspond to serologically defined haplotypes, others have microsatellite designations only, and some are novel haplotypes that have not been validated.

^bND serologic designation not determined. These five horses were tested only using polymorphic intra-MHC microsatellites.

^cData presented in Figures 2A, 3A, and 7A.

^dData presented in Figures 2B, 3B, and 7B.

^eData presented in Figures 2C, 3C, and 7C.

^fData presented in Figures 2D, 3D, 7D, and 8, B and E.

^gData presented in Figures 2E, 3E, 7E, and 8, C and F.

^hData presented in Figures 2F, 3F, and 7F.

ⁱData presented in Figures 2G, 3G, and 7G.

^jData presented in Figures 2H, 3H, and 7H.

^kThis recipient was confirmed not to be of the A2 haplotype, and therefore incompatible with the donor sire, by testing with a subset of the microsatellite panel.

lifespans of 75 days for the primary transplants and 67 days for the secondary transplants (Fig. 5).

In summary, a prolonged break in exposure did not appear to alter the lifespan of subsequent trophoblast transplants. Transplants placed immediately after the rest period, and transplants placed as the eCG once again approached baseline, had median lifespans within the range of invasive trophoblast tissue in normal equine pregnancy, primary transplants in naive recipients, and secondary transplants in recipients with primed immune systems in our core experiment.

Ectopic Trophoblast Transplants Can Survive in the Absence of Progesterone

Serum progesterone was tracked following all trophoblast transplantations to assess the status of the recipient's reproductive cycle (Figs. 6, E and F; 7; and 8, D–F). The experimental plan was designed to confine the serial transplants to the normal horse breeding season in the northern hemisphere, approximately March through November. How-

ever, the longer lifespans of some transplants resulted in experiments that extended into the late fall and winter. This produced unexpected observations. First, the presence of serum eCG from ectopic trophoblast did not prevent some recipients from entering a period of anestrus during the winter months. Second, the continued presence of progesterone was not required for the survival of the ectopic trophoblast. In recipient 3875 (Fig. 8, A and D), the third transplant was placed after an anestrus period had started. The conceptus used for this transplant resulted from the breeding of sire 3474 to donor mare 3725 with the A2/? haplotype. Recipient 3919 entered anestrus toward the end of her secondary transplant (Fig. 8, B and E), and in recipient 4273 a period of anestrus occurred in the middle of the secondary transplant (Fig. 8, C and F).

In summary, these findings demonstrate that the uninterrupted presence of progesterone is not required for trophoblast transplant survival, and that the presence of eCG is not adequate to manipulate the normal seasonal anestrus of the mare.

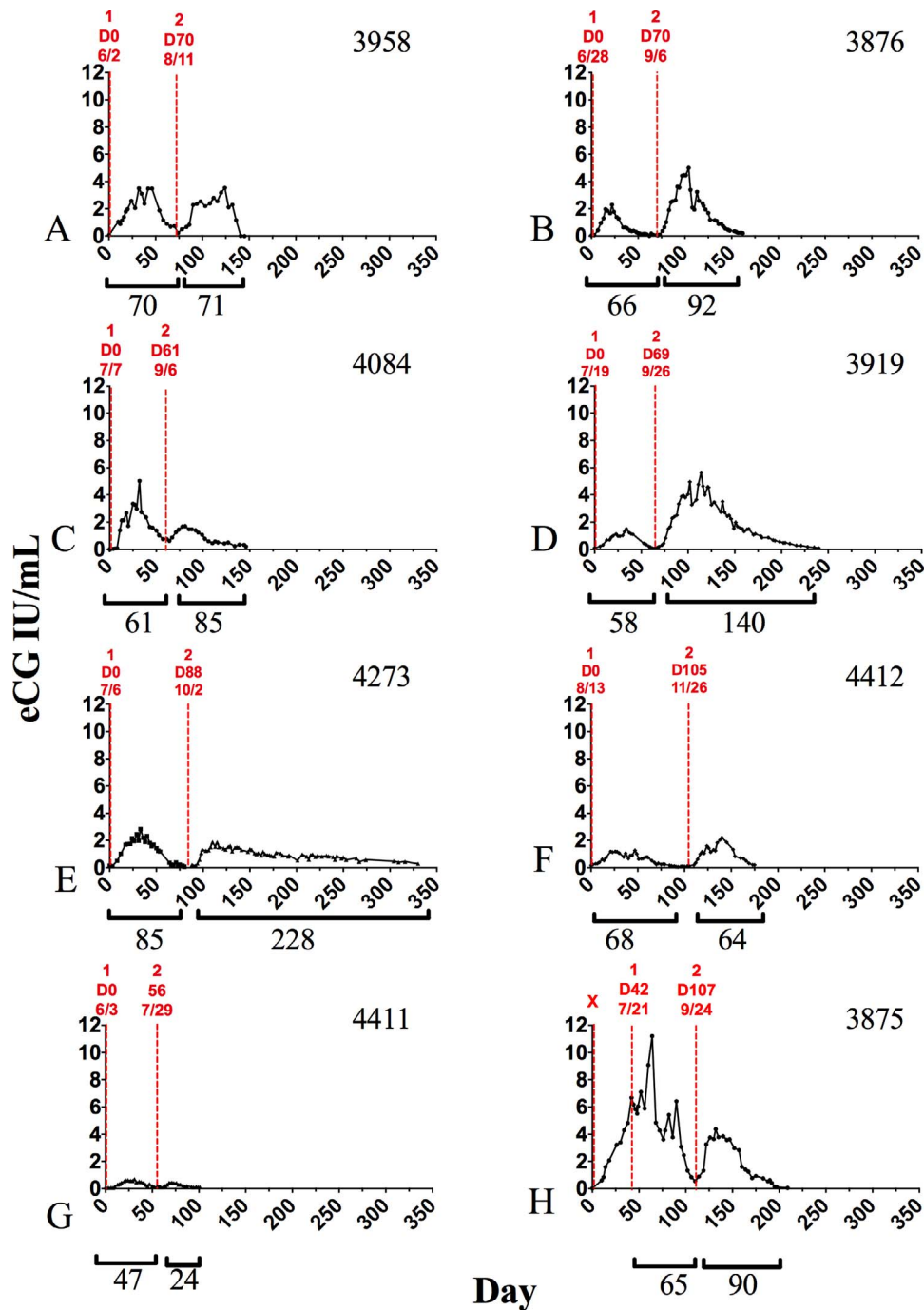


FIG. 2. A–H) Serum eCG curves generated by paired serial ectopic trophoblast transplants in eight recipient mares. Eight mares received two serial ectopic trophoblast transplants (from the experiments indicated in Fig. 1a). Equine chorionic gonadotropin was tracked in the recipients' serum following each transplant using a commercial ELISA assay. Both primary (red dashed line labeled 1) and secondary (red dashed line labeled 2) transplants produced detectable eCG. The eCG curves were used to calculate transplant lifespan. The day of experiment, and the month and day the transplant was performed, are also indicated. The calculated lifespan of each transplant is shown below its x-axis.

DISCUSSION

The mechanisms by which pregnancy induces immunologic memory and how that memory affects subsequent pregnancies are largely unknown. Understanding the role of immunologic memory in the complex set of physiological adaptations that ensure maternal tolerance of the fetal-placental unit is prerequisite to defining its role in reproductive pathology. This is particularly important when considering monotoxic, monogamous species, such as the

human, in which two successful pregnancies per female are required to maintain stable population numbers. With pair bonding of long duration, human females commonly carry two or more pregnancies of similar antigenic character. Dysregulation of immunologic memory in this scenario could compromise fetal survival [29].

Our investigation of trophoblast-induced immunologic memory in another monotoxic species, the horse, showed that there is no significant difference between the lifespans of primary and secondary ectopic trophoblast transplants, even

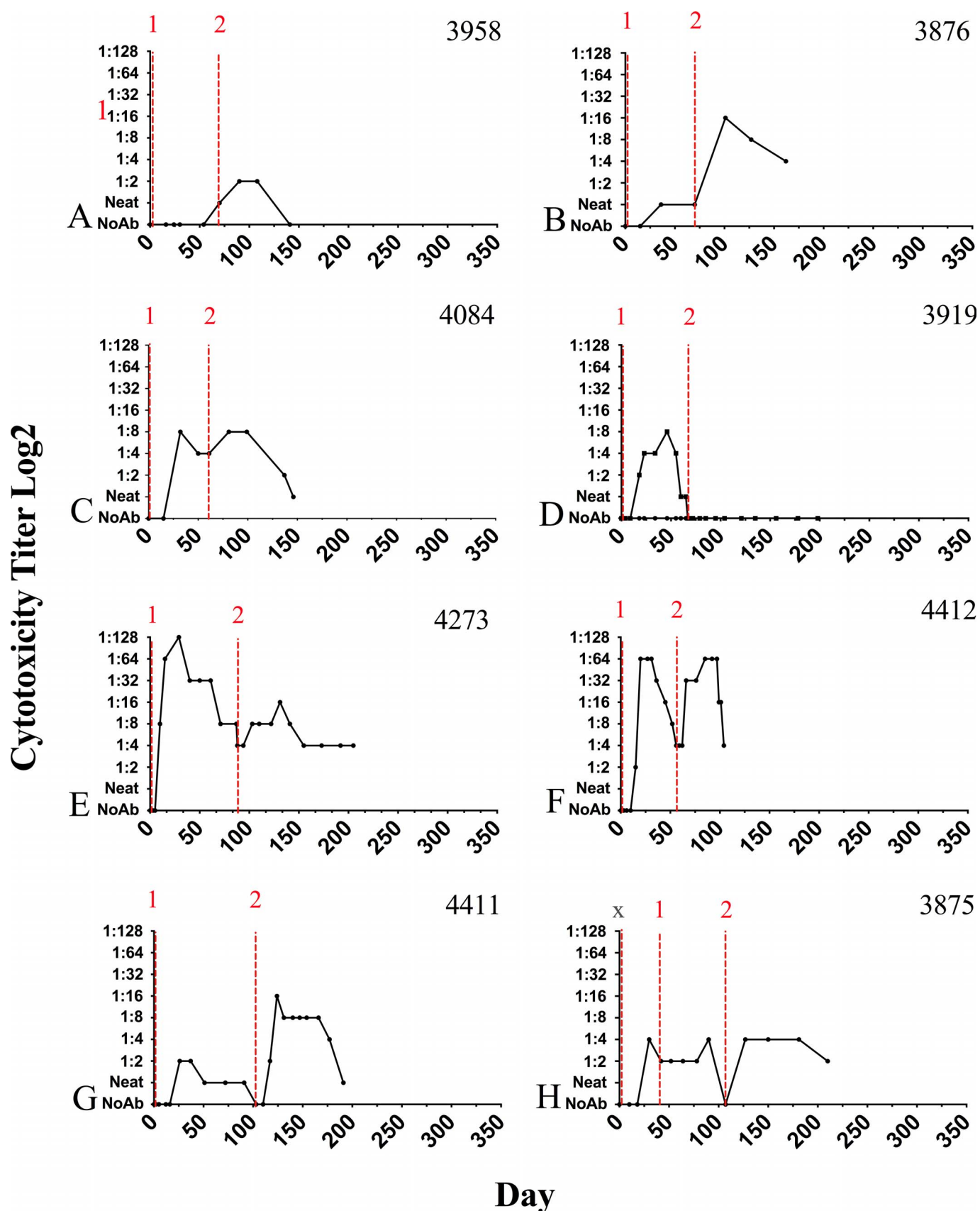


FIG. 3. A–H) Cytotoxic antibody titers produced by recipient mares in response to primary and secondary ectopic trophoblast transplants. Cytotoxic antibody production by recipient mares (from the experiments indicated in Fig. 1a) was determined using a lymphocyte cytotoxicity assay. All recipients' sera were free of cytotoxic antibody against donor animals at the start of the experiment. All primary (red dashed line labeled 1) transplants resulted in cytotoxic antibody production against the MHC-I of the incompatible donor sire or dam by the recipient mare. Mare 3919 (D) was compatible with the donor sire and did not produce antibodies against the sire's MHC-I, but it did produce antibodies against the incompatible donor mare of the primary transplant. Five of the eight mares still had circulating antibodies at the time the secondary (red dashed line labeled 2) transplants were performed. Mare 3919 did not produce antibodies against the compatible donor mare of the secondary transplant.

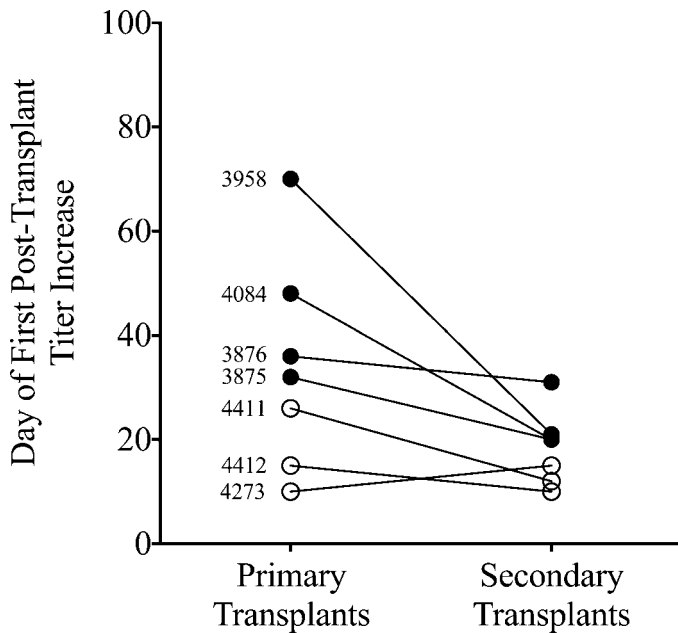


FIG. 4. Length of time to increases in serum cytotoxic antibody titers following primary and secondary ectopic trophoblast transplants. Cytotoxic antibody titers were determined using a lymphocyte cytotoxicity assay. The first day that an increase in antibody titer was detected following primary and secondary transplants is plotted for each recipient that received MHC-I-incompatible transplants. Six of seven antibody responses occurred more rapidly in secondary transplants compared with primary transplants, consistent with a memory response. Open circles indicate transplants from conceptuses sired by the ELA-A2 homozygous stallion, and dark circles indicate transplants from conceptuses sired by the ELA-A3 homozygous stallion.

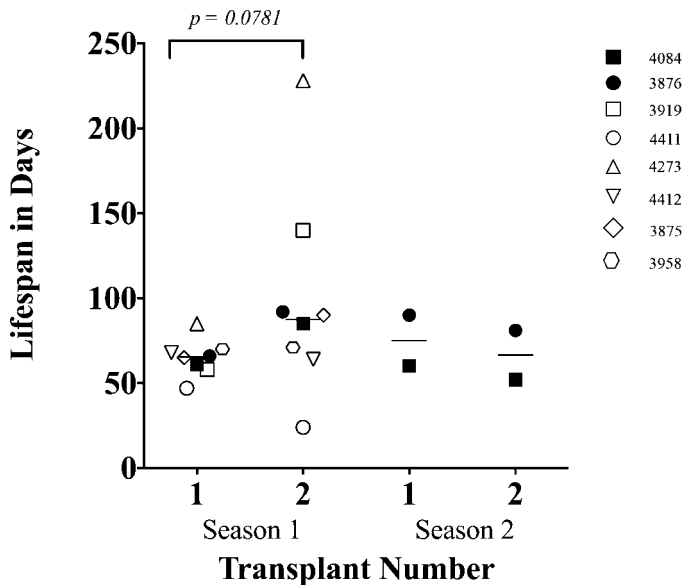


FIG. 5. Lifespans of primary and secondary transplants during two breeding seasons. The lifespans of all paired transplants are plotted. Each symbol represents an individual animal as indicated in the legend. Horizontal bars indicate medians. During the first breeding season there was no significant difference ($P = 0.0781$) in the median lifespan of primary versus secondary transplants in the eight mares as determined using the Wilcoxon signed-rank test. Primary transplants had a median lifespan of 66 days, whereas secondary transplants had a median lifespan of 88 days. An additional set of paired transplants during the second breeding season showed comparable lifespans, with medians of 75 and 67 days, respectively.

with clear anamnestic recognition of secondary transplants. This finding does not support our hypothesis that trophoblast lifespan would be significantly altered when transplanted into a recipient with a primed compared with a naive immune system. There are two possible explanations for the observed uniformity in transplant lifespans. First, it is consistent with a paradigm of protective memory in pregnancy [29, 30] that would support early survival of the MHC-I-positive secondary trophoblast transplants, implying that the immune responses to primary and secondary invasive trophoblast transplants may differ in our experimental system. This may by extension also hold true for invasive trophoblast in normal horse pregnancy. Second, equine invasive trophoblast may possess an intrinsic ability to evade immune destruction via a mechanism that functions independently of the recipient's prior exposure.

Induction of protective immunologic memory has been documented in pregnant females of other species [31]. First pregnancies in mice induce development of fetal-specific memory T-regulatory cells that protect fetuses in later pregnancies. When these cells are partially ablated, fetal resorption is decreased in mice carrying their second pregnancies compared with mice carrying their first pregnancies, consistent with the presence of a population of memory cells [21]. Second pregnancies in humans have been associated with lower risk of preeclampsia, but only when paternity is the same. This suggests increased tolerance to paternal antigens resulting from immunologic memory induced by first pregnancies [32]. Pregnancy-induced tolerance can be non-tissue-specific and enduring. Multiple syngeneic pregnancies in mice resulted in tolerance to skin grafts expressing the male-specific H-Y antigen [20].

Our findings are remarkable in that we did not use pregnant animals in this study, rather only ectopic invasive trophoblast in nonpregnant recipients. Our results differ from those of other studies that used ectopic trophoblast tissue in nonpregnant recipients. Results in these studies were more similar to conventional transplants. When wild-type trophoblast was injected into the tail vein of mice, 99% was cleared by 24 h after administration [33]. Intradermal and renal subcapsular trophoblast transplants begin to die rapidly after 5 to 7 days, although a small percentage may linger to 14 days [19, 34]. Survival times only approached 3 mo when trophoblast stem cells were used, and were injected into an immune-privileged site [35]. Trophoblast transplants placed in pregnant rodents survived longer than those in nonpregnant recipients [34], again suggesting that intact pregnancy confers tolerance.

Studies investigating the effect of immunologic priming on ectopic trophoblast survival generally demonstrate an accelerated destruction of secondary transplants consistent with classical immunologic memory. In rodent recipients primed with skin grafts and splenic cell injections, trophoblast transplants generally failed to survive as long as those in naive recipients [36, 37]. When immune priming was achieved by two sequential ectoplacental cone transplants, transplant cross-sectional area and cell nuclei counts were significantly reduced in secondary transplants compared with primary transplants at Days 5, 7, and 12 after transplantation [19].

Our study, by contrast, clearly demonstrated the long-term survival of strongly MHC-I-positive, freshly harvested, secondary trophoblast transplants in nonpregnant recipients with primed immune systems, and in an ectopic site that was not immune privileged. The lifespans of our primary and secondary transplants were similar to trophoblast tissue survival in normal horse pregnancy. The 88-day median survival time of our secondary transplants was comparable to the 60- to 90-day range reported for endometrial cups in normal

TABLE 2. Season 2 paired serial trophoblast transplant recipient and donor mares and stallions.^a

Parameter	Recipient	Donor stallion	Transplant 1 donor mare	Transplant 2 donor mare
Horse ID #	3876 ^b	3474	3875	3908
Serological ELA haplotype(s)	A2/?	A3/A3	A2/?	A2/A2
MHC microsatellite haplotype(s)	A2/novel	A3b/A3c	A2/novel	A2/A2
Year of birth	2006	1999	2006	2007
Horse ID #	4084 ^c	3474	3875	3725
Serological ELA haplotype(s)	A5/A9	A3/A3	A2/?	A2/?
MHC microsatellite haplotype(s)	novel/novel	A3b/A3c	A2/novel	novel/novel
Year of birth	2006	1999	2006	2003

^aELA (MHC) Haplotypes: the two donor stallions were bred for MHC class I homozygosity at the ELA-A2 and ELA-A3 haplotypes. Other serological (ELA) designations are shown when known. ? indicates MHC haplotype for which no identifying alloantisera have been developed. Some microsatellite haplotypes correspond to serologically defined haplotypes and some are novel haplotypes that have not been validated.

^bData presented in Figure 6, A, C, and E.

^cData presented in Figure 6, B, D, and F.

horse pregnancy [38], to the 75 days for single ectopic horse trophoblast transplants reported in previous studies [18], and to the 66 days for primary transplants in the current study.

The survival of our secondary transplants raises questions as to the biologic mechanisms involved. Although the noninvasive design of our study precluded analysis at a molecular level, we can speculate in the context of existing research in horses and other species. We propose that the protective mechanism is rooted in cellular immunity. Studies in rodents have demonstrated that cellular immunity rather than humoral immunity plays an important role in the transfer of pregnancy-induced tolerance. Transfer of immunity against ectopic blastocyst transplants was attained by adoptive transfer of lymphocytes but not serum [37]. Tolerance to conventional male skin grafts induced by multiple syngeneic pregnancies can be transferred to nulliparous mice via splenic T cells or thymocytes [20]. Mares carrying MHC-I-incompatible pregnancies generate cytotoxic antibodies against paternal antigens. They show evidence of immunological memory upon subsequent pregnancies of the same antigenic character, with a more rapid and stronger antibody response following trophoblast invasion [15]. However, experimental manipulation of the antibody response has shown no evidence that the antibodies have an effect on survival of the endometrial cups arising from the invasive trophoblast [39, 40]. Our single compatible transplant did not live indefinitely, further indicating that the antibodies played no role in determining transplant lifespan.

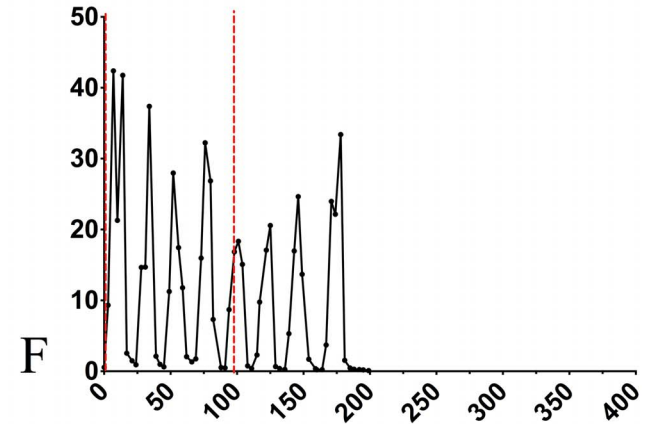
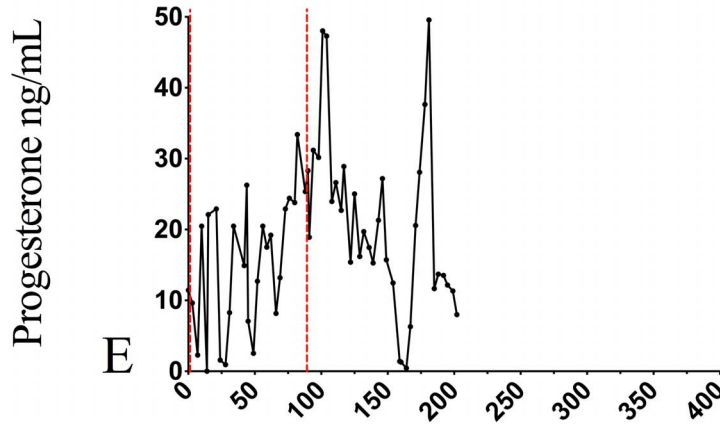
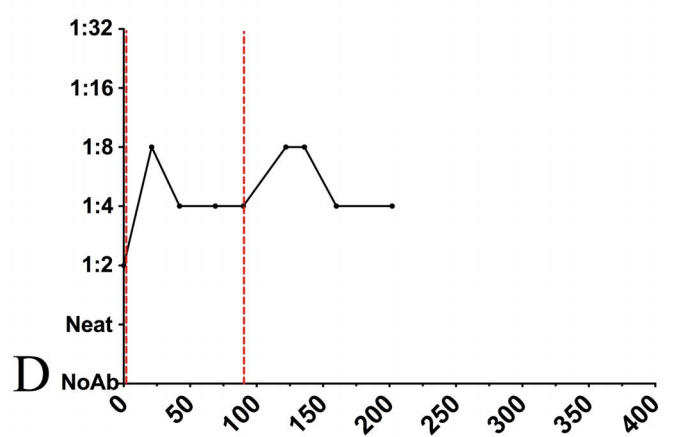
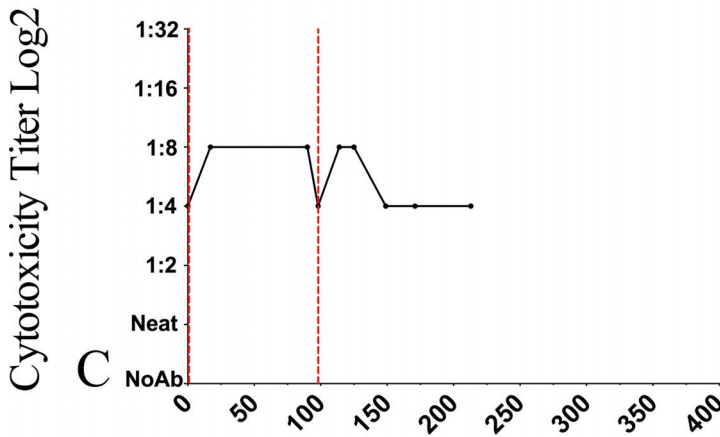
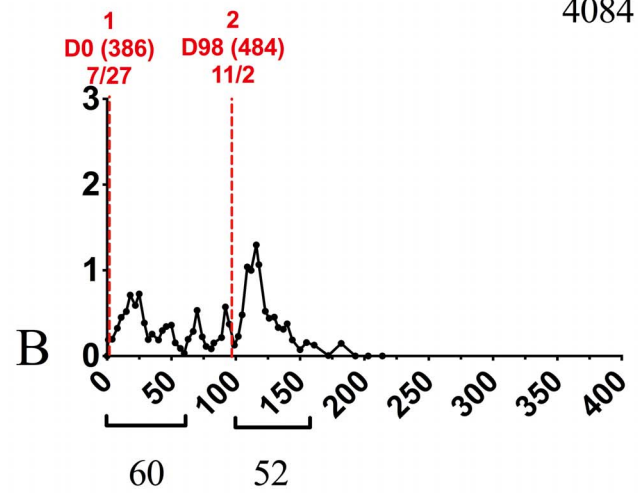
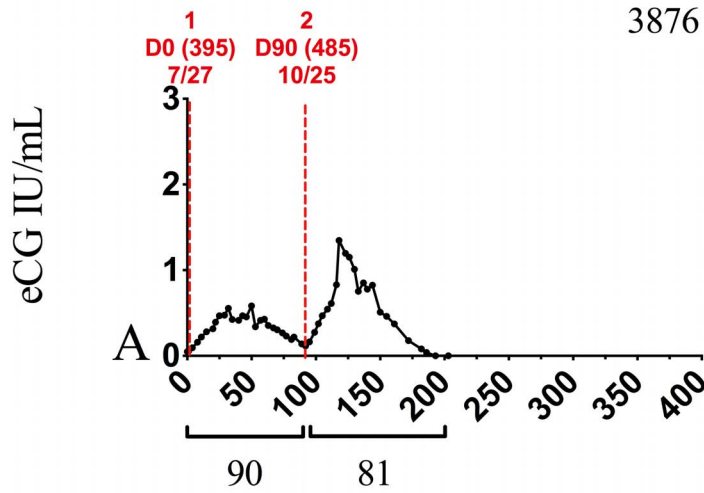
Research suggests a significant role for regulatory T cells in fetal tolerance in human pregnancy [41, 42]. Higher numbers of regulatory T cells are present in decidua compared with peripheral blood. Fetal-specific T-regulatory cells may migrate to the decidua to regulate immune activity at the maternal-fetal interface [43, 44]. In mice, fetal-specific T-regulatory cells generate memory pools following first pregnancies, and provide an additional level of protection to subsequent pregnancies [21]. Evidence exists for T-regulatory cells in association with invasive trophoblast in normal horse pregnancy. Invasive trophoblast forms the endometrial cups upon migration into the uterine stroma. The cups are immediately surrounded by T-lymphocytes but are not destroyed. The infiltrating cells include a higher percentage of FOXP3⁺ CD4⁺ T-regulatory cells relative to peripheral blood [23]. Mares with early pregnancy loss have lower levels of peripheral blood T-regulatory cells [22]. It is reasonable to hypothesize that memory T-regulatory cells generated in response to our primary transplant ensured early survival of the MHC-I-positive secondary transplant. It is of note that our longest surviving secondary transplant was in the recipient with a history of pregnancy. It may be that a pool of pregnancy-

induced memory T-regulatory cells conferred additional protection to this graft. Because the sire of the pregnancy had a different MHC-I haplotype than the sire of the conceptus used for the transplant, the memory cells would be MHC-I nonspecific in nature.

Alternatively, the extended secondary transplant survival in our study may not be dependent on immunologic memory induced by the primary transplant. Rather, the lifespan of both primary and secondary transplants may depend on an intrinsic ability of the trophoblast to manipulate the recipient's immune response via a mechanism that is independent of prior exposure. One possibility is systemic, nonspecific dampening of the cellular immune response. The maternal immune response in equids has been characterized as a "split tolerance," in which the humoral immune system responds to incompatible pregnancies by producing cytotoxic antibodies, whereas the T-cell response in all pregnancies is dampened systemically, as evidenced by a generalized decrease in peripheral blood cytotoxic T-lymphocyte (CTL) reactivity [45, 46]. Similarities may exist between MHC-I-expressing invasive trophoblast and HLA-G⁺ extravillous trophoblast of humans [47], whereby local regulatory T cells are thought to be induced by conversion at the maternal-fetal interface.

The pregnancy hormones progesterone and chorionic gonadotropin are inconsistently implicated in the immunomodulatory events of both gestation [48–51] and ectopic trophoblast survival [34, 35]. Our results suggest that continuous exposure to progesterone is not required for ectopic trophoblast survival. Recipient mares did not undergo the month of progesterone priming as occurs in normal horse pregnancy prior to trophoblast invasion, although some recipients were in diestrus at the time of transplantation, and all recipients produced progesterone in response to eCG. Three recipients entered seasonal anestrus during the experiment. Serum progesterone was undetectable for up to 95 days, yet the trophoblast transplants continued to survive. It is possible that the influence of progesterone is via a secondary molecule, as with uterine milk protein in sheep [52], or that transient exposure was adequate to initiate a protective mechanism. One notable difference between the trophoblast tissue used in our study and that used in rodent studies is its secretion of eCG. Unlike human chorionic gonadotropin [49], a definitive immunosuppressive role for eCG in equine pregnancy has not been established, but it could impact transplant survival via an as of yet undefined mechanism. In fact, the three mares that entered anestrus had the longest surviving secondary transplants. Progesterone deficiency caused by ovariectomy increases CD4⁺FoxP3⁺ peripheral blood lymphocytes in some

3876



Day

FIG. 6. Paired serial transplants performed during the second breeding season. Two of the original eight mares received an additional set of paired transplants the following breeding season (as indicated in Fig. 1c). Equine chorionic gonadotropin was detected in the recipients' serum following both primary and secondary transplants (A and B). Both mares still had circulating cytotoxic antibody at the time of the primary transplant (C and D). Both transplants in recipient 3876 were followed by a period of prolonged diestrus (E), whereas neither transplant in recipient 4084 produced this effect.

rats [53]. This does not appear to hold true in normal anestrus mares [54], but it could have affected the immune response to ectopic trophoblast.

Our study did have some limitations, most notably the seasonally anestrus character of the mare's reproductive cycle, and the natural biologic variation in invasive trophoblast

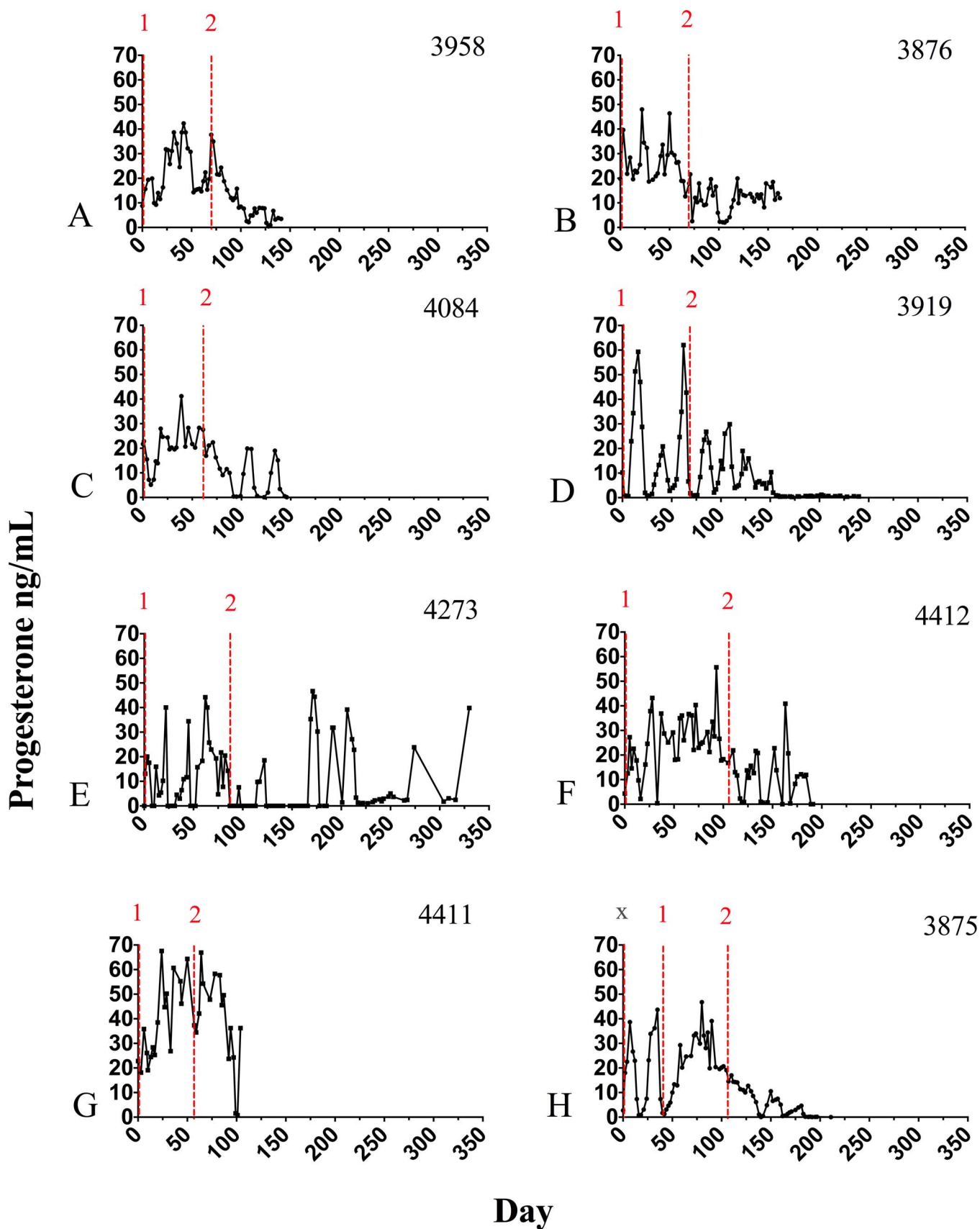


FIG. 7. A–H) Serum progesterone concentrations following primary and secondary transplants. Serum progesterone was monitored following all transplants (from the experiments indicated in Fig. 1a) using a commercial ELISA assay. This allowed determination of the status of the recipients' reproductive system. Mares 3919 and 4273 experienced periods of anestrus during their secondary transplants.

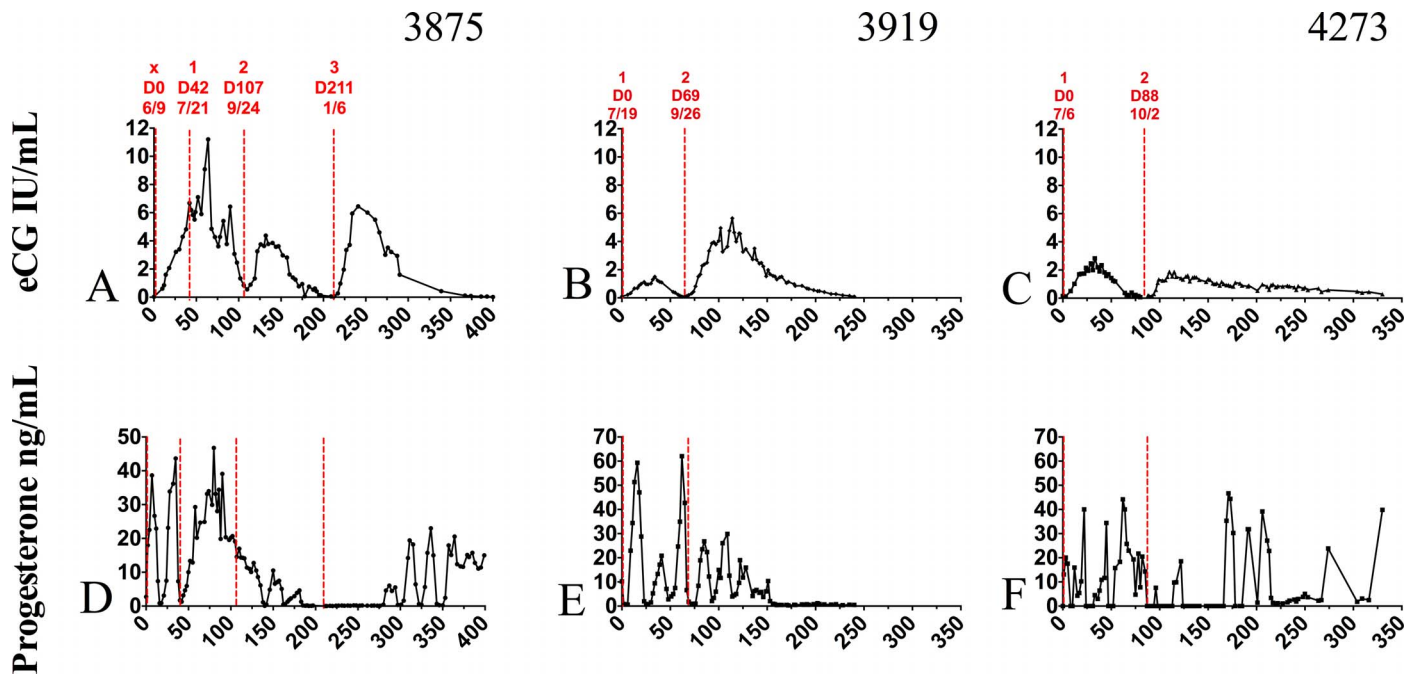


FIG. 8. Trophoblast transplant survival in the absence of progesterone. A subset of mares in this study showed periods of seasonal anestrus while transplants were alive and functional. Following the paired-transplant study in recipient 3875, an additional transplantation was performed in January after the mare had already entered an anestrus period. The transplant remained functional for 156 days (A), and the mare did not begin normal estrus cycles until March (D). Recipient 3919 entered anestrus at the end of her secondary transplant (B and E), and recipient 4273 had a period of anestrus in the middle of the transplant life cycle (C and F).

survival. These factors likely accounted for much of the variation in lifespan of secondary transplants. In mares bred to the same stallion several years in a row, a decrease in the period of eCG production is sometimes observed [55]. In the pathological condition “persistent endometrial cups,” trophoblast cells can survive for more than a year, failing to deteriorate for reasons that are not yet understood [56]. Other causes of lifespan variability may have included immunologic phenomena not specific to reproduction, such as anergy, exhaustion, or senescence from chronic antigenic exposure [24]; partial technical failure; or biologic variation in chorionic girdle size and quality.

We accounted for some of this variability through our statistical approach. In our original sample-size justifications, we confirmed 90% power for detecting a difference of 14 days between mean survival times in primary and secondary transplants, with eight mares. We selected 14 days as the target minimum difference to detect based on the variability of normal equine pregnancy. Sample-size formulae assume parametric distributions. In fact, the distributions of the differences in survival were right-skewed, and we therefore used nonparametric methods. We would expect slightly less power for the nonparametric analysis required by the skewed data, or alternatively, the same power for a slightly larger difference.

We have clearly demonstrated that ectopic trophoblast transplants in the horse are refractory to destruction by secondary immune responses, a finding consistent with previous studies that failed to induce significant alteration of endometrial cup lifespan in the context of equine pregnancy [39, 40]. It is known that equine trophoblast is not inherently immune privileged [57], but the noninvasive approach required to determine lifespans prevented us from investigating specific immune mechanisms responsible for transplant survival. Future experiments using CTL assays and transplant site biopsies

would identify mechanisms of the local and systemic responses to ectopic trophoblast, and identify differences in the response to primary and secondary transplants. Use of the ectopic trophoblast transplant model in ovariectomized and multiparous mares would clarify the significance of our incidental findings regarding the roles of progesterone and prior pregnancies.

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