Suppression of lymphocyte proliferation by a > 30,000 molecular weight factor in horse conceptus-conditioned medium

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Suppression of Lymphocyte Proliferation by a >30 000 Molecular Weight Factor in Horse Conceptus-Conditioned Medium


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ABSTRACT

In this experiment we have identified and partially characterized the immunosuppressive activity of preimplantation horse conceptus-conditioned medium (HCCM). Horse conceptuses were nonsurgically flushed from mares at Days 9-10 (n = 6), 15-16 (n = 3), and 25-26 (n = 3). After incubating the conceptuses for 24 h in RPMI-1640 supplemented with 15% fetal calf serum (FCS) and 1% penicillin/streptomycin, HCCM was obtained from cultures and tested for immunosuppressive activity in lymphocyte proliferation assays. Peripheral blood lymphocytes obtained from randomly selected mares were stimulated with mitogens ( pokeweed mitogen [PWM], concanavalin A [Con A], and phytohemagglutinin [PHA]) in cultures supplemented with 0%, 25%, or 50% HCCM. HCCM from all cultures suppressed lymphocyte proliferation induced by all three mitogens (p < 0.001). After being subjected to various treatments (heating, freeze-thawing, and nitrocellulose filtration), HCCM maintained its full biological suppressor activity. Amicon microconcentrators with 10 000 and 30 000 molecular weight (MW) exclusion filter membranes were used to fractionate HCCM by molecular weight. The suppressor factor was found to be in the >30 000 MW fraction. HCCM was further tested interspecifically on donkey and goat lymphocytes stimulated with PWM. HCCM did suppress proliferation of interspecific lymphocytes (p < 0.01); however, the suppressive capacity of HCCM in caprine lymphocyte cultures was less (p < 0.05) than that observed in equine cultures. These data support the hypothesis that the horse conceptus produces an immunoregulatory factor. This factor is extremely stable and appears to exhibit some degree of species-specificity. The production and immunosuppressive effectiveness of such a factor may play an important role in maintaining the fetal allograft throughout gestation.

INTRODUCTION

The mechanism by which the fetal allograft escapes maternal immunological attack during pregnancy remains largely unknown. It is well documented that fetal tissue in intimate contact with uterine epithelium expresses antigens early in development (Chatterjee-Hasrouni and Lala, 1981; Pavia et al., 1981; Allen et al., 1986). The maternal immune system recognizes the fetal antigens and frequently responds with a humoral immune response. Antibodies against fetal antigens are present in the maternal circulation of up to 90% of pregnant mares (Allen et al., 1987). A cell-mediated immune response also seems to occur at the fetal-maternal interface, and may even be essential for proper implantation (Beer et al., 1975). These immunological events have no detrimental effects on the fetus or placenta of natural intraspecific pregnancies. Yet with many interspecific or hybrid pregnancies, the protective mechanism normally involved during gestation seems to be ineffective, and abortion generally occurs (McGovern, 1975; Clark et al., 1984).

The production of chimeric embryos, resulting in a placenta derived mainly from an embryo of the recipient maternal species and the fetus originating from a different species or hybrid embryo, makes it possible to produce offspring from recipients that will not otherwise carry the fetus to term (Rossant et al., 1982; Fehilly et al., 1984; Polzin et al., 1987; Roth et al., 1989). During such chimeric pregnancies, antibody titers against xenogeneic and allogeneic antigens have been identified in maternal circulation, yet the fetus remains unharmed and is successfully carried to term (Ruffing et al., 1988; MacLaren et al., 1990). Hence, it seems possible that the placenta plays a role in blocking maternal immunological attack (but not recognition) of the fetus, and that the placenta must be of the maternal species in order to efficiently perform this protective mechanism.

Interest in the potential role of the conceptus in regulating maternal immune function has led to the identification of conceptus-derived products with immunoregulatory functions in several domestic species (Clark et al., 1986; Murray et al., 1987; Croy et al., 1988; Pandian et al., 1988). Some products have been identified as interferons (Cross and Roberts, 1989) and are responsible for maternal recognition of pregnancy (Roberts et al., 1985). Others have been identified as immunosuppressive factors when tested in vitro, but their exact mechanism of action or significance in vivo has yet to be elucidated. If these suppressor factors play a critical role in protecting the conceptus from im-
munological attack, then their inability to act effectively across species' lines may provide an explanation for early loss of interspecific pregnancies. In this study we have identified an immunosuppressive substance produced by the preimplantation horse conceptus and have determined some of its characteristics. Furthermore, we have examined the suppressive activity of this substance on lymphocytes of other species.

**MATERIALS AND METHODS**

**Animals and Embryo Collections**

Fifty-five mares of various lighthorse breeds were teased daily by stallions to identify those in estrus. Estrous mares were artificially inseminated with fresh, raw semen (5 × 10⁶ progressively motile sperm) on the second day of estrus and every other day thereafter until ovulation (determined by daily palpation and/or ultrasonography). Conceptuses were collected at three stages of gestation: Group I, Day 9-10; Group II, Day 15-16; and Group III, Day 25-26 (ovulation = Day 0). A total of six Group I conceptuses were collected from six different mares by the nonsurgical embryo collection procedure previously described by Imel et al. (1981). Ultrasonography was used on Day 12 to determine pregnancy in Groups II and III conceptus donor mares. Three Group II and III conceptuses from six different mares were recovered by a modified nonsurgical uterine flushing technique. Mares were lightly sedated with 1–3 ml Rompun (Mobay, Shawnee, KS) administered i.v. 5 min prior to initiating the flushing procedure. The mare's cervix was mechanically diluted by digital palpation. Sterile tygon tubing (18 mm i.d.) was inserted into the vagina and manipulated through the dilated cervix into the uterine body. Throughout the procedure the cervix was held firmly around the tubing to maintain the tubing's position and to prevent fluid leakage from the uterine body out of the relaxed cervix. Sterile PBS supplemented with 1% antibiotic/antimycotic and 1% calf serum was poured into the external end of the tubing. Gauze was placed over the exposed end of tubing and air was blown in, forcing the fluid into the uterus. This process was repeated until no more fluid would enter the uterus. The gauze was removed and the uterine effluent was collected into a sterile flask.

**Conceptus Cultures**

All conceptuses were rinsed under sterile conditions five times with RPMI-1640 (Gibco, Grand Island, NY) supplemented with 15% fetal calf serum (HyClone, Logan, UT; nonheat-treated, <0.1 ng/ml endotoxin) and 1% antibiotic/antimycotic (RPMI+) and were placed in tissue culture flasks containing RPMI+ as follows: Group I, two embryos/4 ml; Group II, one conceptus/12 ml; Group III, one conceptus/30 ml. All cultures were incubated at 37°C in a humidified atmosphere containing 5% CO₂ and air. RPMI+ cultured alone served as control medium (CM). Cultures were terminated at 24 ± 1 h. Horse conceptus-conditioned medium (HCCM) was obtained from cultures, centrifuged at 1500 x g for 20 min, and filtered through a 0.2-μm filter.

**Lymphocyte Proliferation Assays**

Whole blood obtained by jugular venipuncture served as a source of peripheral blood lymphocytes (PBLs). PBLs were isolated by ficoll-gradient centrifugation as described by McClure et al. (1978). Cells were resuspended in RPMI+, counted on a hemacytometer, and diluted to a final concentration of 4 × 10⁶ cells/ml. Cultures were carried out in triplicate in 96-well round-bottomed tissue culture-treated plates (Corning Glass Works, Corning, NY). All wells received 1 × 10⁵ cells (25 μl). Stimulated cultures received 25-μl aliquots of RPMI+ containing one of three mitogens: 1 μg phytohemagglutinin (PHA), 1 μg pokeweed mitogen (PWM), or 5 μg concanavalin A (Con A). HCCM from Groups I and II were tested on T cell mitogen (PHA and Con A)-stimulated lymphocytes to ensure that the observed suppression was not PWM- or B-cell-specific. In addition to mitogen, experimental wells received 0, 25, and 50 μl HCCM while control wells received similar quantities of CM. Nonstimulated cultures contained cells in RPMI+ only. All wells contained a final volume of 100 μl. Each of the three HCCM samples in each of Groups I–III was tested on PBLs from 3–4 randomly chosen mares. Cultures were maintained in an incubator at 37°C in a humidified atmosphere of 5% CO₂ and air. [³H]-Thymidine (6.7 Ci/mmol, ICN, Irvine, CA) was added to cultures (1 μCi/well) at 96 ± 2 h; cells were harvested 16 ± 2 h later onto glass fiber filters using a Skatron automatic cell harvester (Flow Laboratories, Rockville MD). Radioactivity on the filters was determined by liquid scintillation counting. Prior to harvest, percent cell viability in control and experimental cultures was determined by trypan blue exclusion.

**HCCM Characterization**

Characterization tests were carried out with a pool of HCCM derived from three 20-day conceptuses collected and cultured for 48 h as described above, each in 20 ml RPMI+. After centrifugation and filtration, the sample was stored at −80°C in 1.5-ml aliquots. RPMI+ alone was cultured and stored identically to serve as CM for all treatments. To further decrease intra- and interassay variability and to increase assay sensitivity, mitogen titer assays were run on PBL donors to determine the optimal mitogen dose for each donor. PWM was serially diluted from 4–0.05 μg/well and three mares responding optimally to the same mitogen dose of 0.2 μg/well served as PBL donors for all subsequent assays with PWM as the stimulating mitogen. For heat treatment (HT), aliquots of HCCM and CM were thawed to room temperature, and a 600-μl sample of each was placed in a sterile glass vial and held in a beaker of water at 90°C for
30 min. Freeze-thaw treatment (FT) consisted of placing 600 μl HCCM and CM each in a 1-ml cryovial and freezing to −80°C, followed by thawing to room temperature for a total of eight cycles. Two other 600-μl samples of HCCM and CM were filtered two times each through a 0.2-μm nitrocellulose filter (NCF). A final 600-μl untreated fresh-frozen (FF) sample of each HCCM and CM was compared to treated samples. Serial dilutions of all HCCM and CM samples were made with RPMI+ and samples were added to lymphocyte cultures at 0.75, 1.56, 3.23, 6.25, 12.5, and 25 μl/well (total well volume = 100 μl). To identify the approximate size of the substance causing suppression, 500-μl samples were spun at 4 500 RPM for 30 min through amicon microcentrators (Amicon, Danvers, MA) with molecular weight (MW) exclusions of 10 000 and 30 000. Sample concentrates were rinsed by diluting with 500 μl RPMI-1640 and spinning again at 4 500 RPM for 30 min. Concentrate was again diluted back to its original 500-μl volume with RPMI-1640. The two MW fractions of HCCM and CM were added to lymphocyte proliferation assays in quantities of 2-, 4-, and 8-μl sample/well to test for suppressive activity.

**Interspecies Assay**

The suppressive effects of HCCM on goat, donkey, and horse lymphocytes was compared. PWM-titer assays as described for horse PBL donors were used to screen potential goat and donkey PBL donors. Three does and three jennies with optimal responses at 0.2 μg/well PWM were chosen for the interspecies assay. PWM-stimulated lymphocyte proliferation assays were set up as described previously with lymphocytes from the three chosen PBL donors of each species and with HCCM or CM added at 0.4, 0.8, 1.6, 3.2, 6.4, and 12.8 μl/well (total well volume = 100 μl).

**Statistical Analysis**

Average cpm and percent of stimulated control (% of control = average cpm of experimental cultures/average cpm of stimulated control cultures × 100) for triplicate or quadruplicate wells were determined. Results (cpm) from the addition of HCCM of Groups I, II, and III cultures to lymphocyte proliferation assays were analyzed by ANOVA using the general linear model procedures of the statistical analysis systems (SAS) computer package (Luginbuhl et al., 1985) to determine suppressive activity of HCCM. Linear and quadratic contrasts were used to determine the relationship between the addition of 0, 25, and 50% HCCM and percent suppression. HCCM-induced suppression was compared between groups by Tukey’s mean comparison tests. Results from treated HCCM and interspecies assays were expressed as average cpm and/or percent of stimulated control and were similarly analyzed by ANOVA and Tukey’s mean comparison tests. Polynomial regression analysis was used to compare suppressive activity curves in the inter-species assay.

**RESULTS**

**Groups I–III**

When supplemented to cultures at 25% or 50% of the total well volume, HCCM from Groups I, II, and III suppressed lymphocyte proliferation (p < 0.001) induced by PWM, whereas CM exhibited no suppressive activity (p > .05) when similarly added to cultures (Table 1). In all groups there was a linear relationship (p < 0.01) between percent suppression and the addition of 0, 25, and 50% HCCM. HCCM also suppressed Con A- and PHA-induced lymphocyte stimulation (p < 0.001) when added to cultures at 25% and 50% of well volume. Viability of cells in experimental wells was always 85–95% at the end of the culture period.

**HCCM Characterization**

After treatment (HT, FT, NCF), the addition of increasing volumes of HCCM suppressed (p < 0.05) PWM-induced lymphocyte proliferation in a dose-dependent manner (Table 2). Similarly treated CM did not suppress (p > 0.05) proliferation (data not shown). There was no difference in overall suppressive activity (determined as percent of stimulated control) of HCCM between treatments, nor did suppressive activity of treated samples differ (p > 0.05) from that of nontreated (FF) HCCM (Fig. 1).

The fractions of HCCM separated by 10 000 and 30 000 MW exclusion amicon microconcentrators suppressed (p < 0.01) lymphocyte proliferation, with the larger MW HCCM fraction significantly more suppressive (p < 0.01) than the smaller MW fraction (Figs. 2 and 3). The addition of 8 μl/well of HCCM filtrate and retentate fractions decreased the percent stimulation to 71.3 ± 5.0 (mean ± SEM) and 21.4 ± 1.7, respectively (10 000 MW exclusion centricron), and 77.2 ± 5.1 and 28.4 ± 9.1, respectively (30 000 MW exclusion centricron); the addition of either MW fractions of CM had no suppressive effect on percent lymphocyte proliferation (103.6 ± 5.1 and 118 ± 7.1 [10 000 MW centricron] and 112.3 ± 1.0 and 108.5 ± 7.3 [30 000 MW centricron]).

**Inter Species Assay**

The addition of HCCM to goat, donkey, and horse PWM-stimulated lymphocytes decreased (p < 0.05) lymphocyte proliferation across all species (Fig. 4). Overall suppression (determined relative to stimulated controls) was less in goat lymphocyte cultures than in horse or donkey cultures (p < 0.05), but there was no difference between percent suppression of donkey and horse cultures. Polynomial regression analysis used to describe the dose-response suppression curves resulting from the addition of HCCM to lymphocyte cultures of the three species determined that the curves representing horse and donkey lymphocyte suppression had the same slope and were best fit by a quadratic equation, whereas the curve for goat lymphocyte suppression was best fit by a linear equation.
HORSE CONCEPTUS-DERIVED LYMPHOCYTE SUPPRESSION

Table 1. Effect of control medium and HCCM from Groups I-III on mitogen-stimulated horse PBL proliferation.*

<table>
<thead>
<tr>
<th>Source of medium</th>
<th>Added (%Vol)</th>
<th>Mitogen stimulation (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>None</td>
</tr>
<tr>
<td>Group I HCCM (Day 9-10)</td>
<td>0</td>
<td>6 896 ± 2 299</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Group II HCCM (Day 15-16)</td>
<td>0</td>
<td>35 286 ± 5 377</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Group III HCCM (Day 25-26)</td>
<td>0</td>
<td>14 018 ± 2 194</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Control Medium</td>
<td>0</td>
<td>6 896 ± 2 299</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50</td>
<td></td>
</tr>
</tbody>
</table>

*Group I and control medium data represent mean cpm ± SEM for three Day 9-10 HCCM samples and three CM samples, respectively, each tested on three PBL populations (total n = 9). Groups II and III values are the mean cpm ± SEM from three Day 15-16 HCCM samples and three Day 25-26 HCCM samples, respectively, each tested on four PBL populations (n = 12). HCCM from all groups (I, II, and III) added at 25% and 50% of well volume suppressed lymphocyte proliferation (p < 0.001) in mitogen-stimulated cultures, but CM did not (p > 0.05).

Supplemented to cultures at 1 µg/well.
Supplemented to cultures at 5 µg/well.
Significant lymphocyte suppression relative to stimulated controls (p < 0.001).

Discussion

The data presented herein indicate that the preimplantation horse conceptus produces a factor that suppresses in vitro mitogen-induced horse lymphocyte proliferation. This suppression is not mediated through cytotoxicity. The substance responsible for suppressive activity is released by the embryo as early as Day 9-10 of development and continues to be produced at least through Day 26 of gestation. All horse embryos collected at Day 9-10 had hatched from their zonae and were surrounded by the equine embryonic capsule. Whether or not these embryos produce a suppressor substance prior to hatching from their zonae was not determined. It is possible that the embryo, while surrounded by the fairly non-antigenic zona, is not in danger of immunological attack and thus may not produce a suppressor factor. It has been reported that bovine conceptus-derived suppressor activity is not detectable prior to Day 10, variable from Days 10–12, and only definitely present by Day 14 (Croy et al., 1988). Such reports indicate that, at least in cattle, suppressor substances are not produced and/or released by the embryo prior to zona hatching. It is possible, however, that suppressor factors are produced early but in such small quantities that they are not detected by the in vitro biological assay used to evaluate suppression. Suppressive activity of HCCM varied between, but was definitely present in, the three Day 9-10 cultures. The HCCM with the greater suppressive activity was that from cultures in which embryos remained fully expanded and grew in size during the 24-h culture period. This observation may indicate that the production of the suppressor factor correlates either with the health of the embryo or simply with an increased number of cells capable of producing the substance. It has been proposed that the measure of embryo-derived immunoregulatory function could be used in predicting the viability of embryos prior to their transfer to

Table 2. Effect of treated HCCM on PWM-stimulated horse PBL proliferation.*

<table>
<thead>
<tr>
<th>HCCM added (µl/well)</th>
<th>Fresh-frozen</th>
<th>Frozen-thawed</th>
<th>Heat-treated</th>
<th>Nitrocellulose-filtered</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>70 483 ± 3 844</td>
<td>73 645 ± 5 156</td>
<td>77 985 ± 4 719</td>
<td>39 048 ± 9 774</td>
</tr>
<tr>
<td>0.75</td>
<td>36 370 ± 473</td>
<td>30 281 ± 684</td>
<td>33 383 ± 1 439</td>
<td>21 662 ± 5 735</td>
</tr>
<tr>
<td>1.55</td>
<td>20 959 ± 1 504</td>
<td>21 098 ± 1 587</td>
<td>21 790 ± 1 910</td>
<td>14 645 ± 4 427</td>
</tr>
<tr>
<td>3.125</td>
<td>15 075 ± 1 076</td>
<td>13 400 ± 1 286</td>
<td>9 627 ± 1 817</td>
<td>8 078 ± 2 707</td>
</tr>
<tr>
<td>6.25</td>
<td>8 988 ± 881</td>
<td>11 707 ± 1 007</td>
<td>5 382 ± 1 641</td>
<td>4 424 ± 1 914</td>
</tr>
<tr>
<td>12.5</td>
<td>5 107 ± 457</td>
<td>5 718 ± 556</td>
<td>2 904 ± 362</td>
<td>2 463 ± 1 097</td>
</tr>
<tr>
<td>25.0</td>
<td>3 056 ± 248</td>
<td>3 184 ± 283</td>
<td>1 527 ± 122</td>
<td>1 434 ± 580</td>
</tr>
<tr>
<td>Nonstimulated</td>
<td>14 579 ± 2 208</td>
<td>12 843 ± 3 122</td>
<td>13 986 ± 3 402</td>
<td>2 951 ± 1 316</td>
</tr>
</tbody>
</table>

*Data represent mean cpm ± SEM (n = 3). All samples expressed suppressive activity (p < 0.05) relative to stimulated controls (0.0 µl/well).
FIG. 1. Suppressive activity of treated (heat-treated 90°C for 30 min (HT), filtered twice through a 0.2-μm nitrocellulose filter (NCF), frozen to –80°C and thawed to room temperature eight times (FT)) vs. nontreated fresh-frozen (FF) HCCM on PWM-stimulated PBL proliferation. Columns represent mean % of stimulated control ± SEM (n = 3) with the addition of increasing quantities of HCCM. All samples expressed suppressive activity (p < 0.05), and there was no difference (p > 0.05) between the activity of treated and nontreated HCCM.

FIG. 2. Effect of >10 000 MW and <10 000 MW fractions of HCCM and CM on PWM-stimulated PBL. Fractions were separated by amicon microconcentrator centrifugation. Values represent mean % of stimulated control ± SEM (n = 3). Both HCCM fractions were suppressive compared to CM fractions (p < 0.05). Different superscripts denote significantly different suppressive activity (p < 0.05).

FIG. 3. Effect of >30 000 MW and <30 000 MW fractions of HCCM and CM on PWM-stimulated PBL. Fractions were separated by amicon microconcentrator centrifugation. Values represent mean % of stimulated control ± SEM (n = 3). Both HCCM fractions were significantly suppressive (p < 0.05) compared to CM fractions. Different superscripts denote significantly different suppressive activity (p < 0.05).

FIG. 4. Effect of HCCM on PWM-stimulated PBL of horses, donkeys, and goats. Values are mean % of stimulated control ± SEM (n = 3) of PBL with the addition of increasing quantities of HCCM. Overall suppression of goat PBL was less (p < 0.05) than that of horse or donkey PBL. There was no difference (p > 0.05) between horse and donkey PBL suppression. The goat suppressor curve follows a linear regression equation while the donkey and horse curves follow quadratic equations with the same slope (ANOR; R² = 0.88).

% of stimulated control = average cpm experimental/average cpm stimulated control × 100. Average cpm for goat, donkey, and horse stimulated controls were 122 542, 78 199, and 46 036, respectively.

*Significant difference in overall suppression caused by HCCM supplementation (p < 0.05).
bryos seem to express immunosuppressive activity at this stage of development, it is possible that a measurement of this function could be useful in predicting post-transfer embryo survival. To do so, a quicker, more sensitive assay than lymphocyte proliferation would be required. The difference in suppressive activity of HCCM from Group I vs. Groups II and III is probably due simply to the difference in the tissue:medium ratio in the cultures, resulting in a greater dilution of the Day 9-10 embryo-derived factors.

Because production of the suppressor factor begins as early as Day 9 and continues through Day 26, it is not likely that this substance is responsible for maternal recognition of pregnancy, which occurs between Day 13 and Day 15 (Hershman and Douglas, 1979). Such proteins in other species are produced mainly during the specific window when maternal recognition occurs (Davis and Ott, 1989; Godkin et al., 1989). However, we can not rule out the possibility that the substance plays a role in the event.

Lymphocyte proliferation induced by any of the three different mitogens was suppressed by the addition of HCCM, indicating that the suppressor factor is non-antigen-specific. Furthermore, the use of Con A and PHA (T lymphocyte stimulators) and PWM (a T and B lymphocyte stimulator) indicates that the suppressor factor might affect both lymphocyte subsets. However, because PWM is a T cell-dependent B cell stimulator, it is possible that PWM B cell stimulation is suppressed indirectly by suppression of T cells, which results in decreased lymphokine production (Sharon, 1983; Weiss, 1989). The fact the lymphocyte proliferation was not affected by the addition of CM to the lymphocyte cultures ensures that the suppression observed with HCCM is not an artifact resulting from the use of certain flasks or incubation regimens. Lymphocyte suppression resulting from the incubation of medium in certain flasks or plates has been reported previously (Croy et al., 1988).

The immunosuppressive effect of progesterone and other steroid hormones is well documented, but the level of hormone required to suppress lymphocyte proliferation in vitro exceeds physiological levels. As much as 5 μg/ml is required to achieve suppression of PHA- and Con A-stimulated lymphocyte proliferation (Mori et al., 1977; Siiteri and Stites, 1982). Only picogram-nanogram concentrations of hormones are found in equine blastocoeal fluid or in equine conceptus culture medium (Flood et al., 1979; Mar- san et al., 1987), indicating that—even localized around the conceptus—the hormone levels produced would be far below that required for suppressing lymphocyte proliferation. However, to ensure that the suppression we observed was not hormone-induced, HCCM was run through the amicon microconcentrators with MW exclusions of 10 000 and 30 000. Since the suppressor substance was found to be >30 000 MW, we can conclude that it is not a steroid hormone. The fact that the <30 000 and <10 000 MW fractions were slightly suppressive might indicate the presence of hormones and/or interferons in those fractions of smaller MW.

HCCM was subjected to harsh treatments of freeze-thawing and heating at 90°C for 30 min in an attempt to denature the proteins in the sample. Because no loss of biological activity of the HCCM suppressor factor was observed after treatments, it is possible the substance is not a protein. Alternatively, it may be a protein with a very stable structure, perhaps due to many disulfide bridges. It is also possible the substance does undergo structural denaturation, but that the active sites remain intact or reanneal after treatment. Although the treatments had no effect on the activity of the suppressor substance and the substance was not removed from HCCM when filtered through nitrocellulose, which binds proteins through hydrophobic interactions, we have not yet ruled out the possibility the suppressor factor is a protein. The stability of its biological activity and the fact that the HCCM suppressor factor is >30 000 MW serve as evidence that this equine suppressor substance is different from the proteins that exhibit antiviral activity and that are responsible for maternal recognition of pregnancy in sheep and cows. Both ovine trophoblast protein-1 and bovine trophoblast protein-1 are of the alpha-interferon family (Imakawa et al., 1987; Stewart et al., 1987; Charpigny et al., 1988) and, like other alpha-interferons, are <30 000 MW and unstable when treated with heat or repeated freeze-thawing. A porcine conceptus-derived interferon-like protein with antiviral activity had also been identified; it too is small (22 000–24 000 MW) and extremely labile (Cross and Roberts, 1989). We therefore conclude that the suppressor factor described herein is most likely not of the interferon family.

We have suggested that if the conceptus-derived suppressor substance plays a significant role in protecting the conceptus from maternal immunological assault, the inability of it to cross-react with lymphocytes of another species could in part explain the failure of interspecies pregnancies. It has been shown with sheep and goats that medium conditioned by 20-day-old conceptuses exhibit greater suppressive activity when added to lymphocytes of the same species compared to that seen when added to lymphocytes interspecifically (Roth and White, 1989). The lack of immunosuppressive capacity of conceptus-derived products of one species in the maternal environment of another species could explain the repeated failure of sheep/goat interspecies pregnancies and the apparent evidence of immunological rejection of the conceptus. Horses and donkeys provide a unique model for the study of interspecies cross-reactivity of conceptus-derived substances because both interspecies and hybrid pregnancies are successful. We show herein that there is no difference in overall suppressive activity of HCCM when added to donkey lymphocytes as compared to horse lymphocytes. Thus, it is possible that the horse conceptus-derived suppressor factor does efficiently cross-react with donkey lymphocytes; this may explain, in part, how donkeys are able to carry horse fetuses to term. When added to goat lymphocytes, however, HCCM was much
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