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Conceptus-Derived Immunosuppressive Factors and Their Role in Fetal Allograft Protection.

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CONCEPTUS-DERIVED IMMUNOSUPPRESSIVE FACTORS AND THEIR ROLE IN FETAL ALLOGRAFT PROTECTION

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LIST OF ABBREVIATIONS

CCM - caprine conditioned medium.
Con A - concanavilin A.
CM - control medium.
CTL - cytotoxic T lymphocyte.
CTLL - cytotoxic T lymphocyte line.
CTLL-2 - IL-2 dependent cytotoxic T lymphocyte line.
FACS - fluorescence activated cell sorter.
FF - fresh frozen.
FITC - fluorescein isothiocyanate.
FSH - follicle stimulating hormone.
FT - frozen-thawed 8X to -80° C.
HCCM - horse conceptus conditioned medium.
HCCM-I - horse conceptus conditioned medium with fetal calf serum.
HCCM-II - horse conceptus conditioned medium without fetal calf serum.
HCCM-SF - horse conceptus conditioned medium suppressor factor.
HCM - sheep x goat hybrid conditioned medium.
HMWGP - high-molecular-weight glycoprotein.
HBSS - Hank's basic salt solution.
HT - heat-treated 90°C for 30 min.
HTCM - horse trophoblast conditioned medium.
ICM - inner cell mass.
I-CM - indomethacin control medium.
I-HTCM - indomethacin-treated horse trophoblast conditioned medium.
IL-1 - interleukin-1.
IL-2 - interleukin-2.
IL-2R - interleukin-2 receptor.
rIL-2 - human recombinant interleukin-2.
LPS - lipopolysaccharide.
MHC - major histocompatability.
MLR - mixed lymphocyte reaction.
NCF - nitrocellulose filtered.
NK - natural killer.
OCM - ovine conditioned medium.
PBL - peripheral blood lymphocytes.
PBMC - peripheral blood mononuclear cells.
PBMCS - peripheral blood mononuclear cell supernatant.
PBS - phosphate buffered saline.
PBS-GS - phosphate buffered saline + 10% goat serum.
PHA - phytohemagglutinin.
PGE_2 - prostaglandin E_2
PP14 - placental protein 14.
PWM - pokeweed mitogen.
RIA - radioimmunoassay.
PZP - pregnancy zone protein.
RPMI+ - RPMI-1640 medium + 1% penicillin/streptomycin + 10% calf serum
SIF - suppressor inducer factor.
TCR - T cell receptor.
ULP - uterine luminal protein.
UTM-P - uterine milk protein.

ABSTRACT

The role of conceptus-derived immunosuppressive factors in protecting the fetal allograft was investigated by first examining the immunosuppressive activity derived from ovine, caprine and hybrid conceptus trophoblast tissue. Medium conditioned by 20 day-old conceptus trophoblast tissue from each species suppressed sheep and goat lymphocyte proliferation ($P<.01$). There were no differences ($P>.05$) between the immunosuppressive effects of the three media on either sheep or goat lymphocytes. In subsequent studies, horse conceptus-conditioned medium (HCCM) derived from embryos at days 9, 15 and 26 of gestation, suppressed mitogen-stimulated horse lymphocytes ($P<.001$). HCCM maintained its suppressive activity following heating and repeated freeze-thawing. Using microconcentrator centrifugation, it was determined that the suppressor factor(s) was >100,000 MW. HCCM was tested in donkey and goat lymphocyte cultures and proved capable of suppressing proliferation of xenogeneic lymphocytes ($P<.01$). However, the suppressive capacity of HCCM in caprine lymphocyte cultures was less ($P<.05$) than that in equine cultures. Trophoblast tissue of 21 day-old horse conceptuses was cultured in the presence and absence of indomethacin. Both immunosuppressive activity and the concentration of prostaglandin E_2 (PGE_2) in the medium of each culture were measured. While PGE_2 production was greatly reduced in cultures containing indomethacin, trophoblast-derived immunosuppressive activity was not affected ($P>.05$). The final study involved partial characterization of the HCCM suppressor factor's (HCCM-SF) mechanism of action. By testing HCCM-SF in cultures stimulated with mitogens

that selectively activate B and/or T lymphocytes, and by sorting HCCM-SF-treated and non-treated cells labeled with fluorescent antibodies, it was determined that HCCM-SF acts on T lymphocytes. The temporal effect of HCCM-SF was also investigated and, compared to lymphocytes treated at time 0, proliferation was greater ($P<.01$) in cultures treated with HCCM-SF 24 or 48 hours after the start of stimulation. Furthermore, the removal of HCCM-SF from lymphocyte cultures resulted in complete recovery of cell responsiveness. The addition of recombinant human IL-2 to the lymphocyte cultures did not overcome the suppressive effect of HCCM-SF ($P<.01$), yet HCCM-SF had no effect on cells already expressing the high affinity IL-2 receptor ($P>.05$). HCCM-SF may be one factor responsible for inhibiting cell-mediated fetal allograft rejection during pregnancy.

INTRODUCTION

The mammalian placenta mediates numerous supportive functions related to the fetus that are necessary for maintaining a successful pregnancy. In acting as the organ of fetal respiration, nutrition, and excretion, the placenta facilitates metabolic exchanges between fetal and maternal blood while simultaneously impeding transplacental movement of many other substances. The placenta also plays an important role as an endocrine organ during gestation, but perhaps its most fascinating characteristic is the ability to maintain the fetus as an allograft in the mother.

Scientists have struggled for years to understand how nature circumvents the allograft reaction to allow for fetal survival. The motives for studying this phenomenon have been primarily two-fold. First, it is believed that some cases of infertility and recurrent abortion result from aberrant immunological conditions, but causal, atypical immunological events can only be identified and appropriately treated if the "normal" fetal-maternal relationship of successful pregnancies is understood. Second, information gained from the elucidation of the mechanisms underlying fetal allograft tolerance might prove valuable for those struggling to improve the proficiency of organ transplantation. While both motives still exist today, there is now a third and perhaps more critical incentive for solving the mysteries surrounding maternal acceptance of foreign fetal tissue. This third motive pertains to potential strategies for assisting reproduction of endangered species.

While the human population is expanding at an accelerated rate,

species diversity on this planet is diminishing at a similarly alarming rate. In order to both protect and enhance proliferation of endangered species, it is imperative that some action be taken immediately. With the technological advances of the past decade, interspecific embryo transfer from endangered to non-endangered species has become an attractive strategy. Unfortunately, the use of this procedure has met with only limited success. The etiology of failed interspecies pregnancies frequently indicates immunological rejection. Therefore, it is anticipated that by achieving a comprehensive understanding of the fetal-maternal immunological relationship, it will be possible to either choose more appropriate xenogeneic surrogate females or to modulate the in utero immune response of the surrogates such that the interspecific pregnancy is maintained. Perhaps then, the success of interspecific embryo transfer will attain the efficiency required to facilitate the propagation of some imperiled species.

In this dissertation, one immunological aspect of the fetal-maternal relationship is examined. In order for the reader to gain insight into the philosophy of this work, the literature review in Chapter I encompasses three main topics. The first section describes components of the immune response that contribute to allograft rejection and that may play a role in the acceptance or rejection of the fetal allograft. The second section focuses on the large, convincing body of evidence for the importance of the placenta in both protecting the fetal allograft and maintaining pregnancy. The final section of the literature review deals specifically with the concept

of immunosuppression at the fetal-maternal interface, which is the focus of this research. Chapters II-V consist of the four manuscripts derived from this dissertation.

CHAPTER I.

LITERATURE REVIEW

IMMUNE RESPONSES AND ALLOGRAFT REJECTION

The History of Allografts

An allograft is a tissue that has been transplanted from one member of a species to another genetically distinct member of that species. The first allograft attempt was reported in 1503 by a surgeon who grafted skin from a slave for the reconstruction of the master's nose (from Auchincloss and Sachs, 1989). Skin grafting became common practice during the late 1800s, but it was mainly a technique used to promote the healing of a wound, and whether or not the graft "took", was of no great concern. A tremendous break-through for those studying graft acceptance and rejection in the early 1900's came from the observations of Sir Peter Medawar. Medawar critically evaluated some peculiar results of a clinical skin graft attempt and subsequently carried out a series of grafting experiments in rabbits (Medawar, 1944). Shortly thereafter, Medawar concluded that the rejection of allografts was a form of actively acquired immune reactions (Medawar, 1945). Little (1914, 1941) also made an exceptional contribution towards our understanding of the success or failure of transplantation by describing the "laws of transplantation". These laws dealt with the genetic components contributing to graft rejection. In his interpretation of the complicated laws, Little proposed that there were multiple loci codominantely expressing their products, and if any such product was generated by the donor and not the recipient, the graft would be

rejected (Little, 1914). Little's hypothesis (now considered a fundamental law) was largely responsible for stimulating subsequent investigations that led to the identification and characterization of the major histocompatibility complex (MHC) loci and associated antigens.

Today, we know that the extraordinarily polymorphic MHC antigens (Bell et al., 1985) can be divided into two general types called class I and class II MHC antigens. The class I antigens are found on virtually all nucleated cells of the body (Harris and Gill, 1986). In contrast, class II antigens are selectively present on several immune cells, such as macrophages, dendritic cells, B lymphocytes and human T lymphocytes (Daar et al., 1984). Initially, class I antigens were identified by antibodies. In contrast, class II antigens were identified by their ability to provoke allogeneic lymphocyte responses (Bach et al., 1972). Such responses are not unlike those that occur during allograft rejection.

Humoral Immune-mediated Graft Rejection.

Because B lymphocytes are responsible for the generation of antibodies, their primary role in graft rejection is mediated through a humoral immune response. There is some evidence that pre-existing antibodies can cause antibody-mediated (hyperacute) graft rejection of vascularized organs (Williams et al., 1968). Such rejection is characterized by antibody binding to vascular endothelium which leads to severe thrombosis (Winn, 1986). Chronic antibody-mediated rejection has been implied in a few failed cases of transplantation where only a sparse cellular infiltrate was observed (Jeannet, 1970). However, a well defined mechanism for chronic humoral immune-mediated

graft rejection has not been identified. Furthermore, skin grafts are resistant to hyperacute antibody-mediated rejection (Winn et al., 1973). Instead, cell-mediated immunological processes are responsible for skin graft rejections which occur one or more weeks following transplantation. Because the placental-uterine association is analogous to a skin graft-donor relationship, it is the cell-mediated response, or lack thereof, during pregnancy that receives the most attention when survival of the fetal allograft is studied.

Cell-mediated Graft Rejection.

Skin graft rejection is a highly specific and complex process involving both B and T lymphocytes, macrophages and natural killer (NK) cells (Hayry et al., 1984; Tilney et al., 1984). Experiments in which syngeneic and allogeneic skin grafts were placed adjacent to each other resulted in an inflammatory rejection response to the allogeneic graft, yet the response abruptly ended at the interface of the syngeneic graft (Auchincloss and Sachs, 1989). Furthermore, when mosaic grafts of chimeric (tetraparental) mice were transferred to one of the parents, only allogeneic cells were destroyed while interspersed syngeneic cells were left unharmed (Mintz and Silvers, 1970; Rosenberg and Singer, 1988). The specificity of graft rejection is a reflection of the T cell receptor's (TCR) specificity for foreign cells.

T Lymphocytes

The importance of T lymphocytes in cell-mediated graft rejection has been demonstrated in vitro by their role in non-antibody mediated cytotoxicity, and in vivo by their significant predominance in the cellular filtrate of rejected transplants. Further confirmation has

been obtained experimentally by the demonstration that T cell deficient rats indefinitely accept cardiac allografts (Iga et al., 1985) and athymic mice accept not only allogeneic grafts, but also xenogeneic grafts (Manning et al., 1973). In addition, when such mice are re-populated with purified T cells, the animals regain the capacity to reject grafts (Sprent et al., 1986; Rosenberg et al., 1987). While it is widely accepted that T cells are largely responsible for graft rejection, there is still controversy regarding the specific T cell subsets or mechanisms involved (Steinmuller, 1985; Mason and Morris, 1986).

T cell development is accompanied by changes in cell surface structures and these surface antigens serve as phenotypic markers for cells with different physiological functions. The T cell population is divided into two general sub-populations on the basis of the expression of the specific cell surface antigens, CD4 and CD8 (Reinherz and Schlossman, 1980; Raff, 1971; Cantor and Boyse, 1975). Expression of the CD4 and CD8 antigens is associated with the function of the cells. Thus, CD4+ cells are known as T helper cells and CD8+ cells are T cytotoxic/suppressor cells. CD4 and CD8 antigens are also involved in and predict the TCR's mechanism of antigen recognition. The TCR of CD4+ cells recognize antigen in the context of class II MHC while the TCR of CD8+ cells recognize antigen in the context of class I MHC (Swain, 1983). Reconstitution of T cell-deficient mice with either CD4+ or CD8+ T cell populations re-establishes the animal's ability to reject grafts (Wheelahan, 1987; Auchincloss et al., 1988). It has been reported that rejection of grafts expressing only class I antigenic differences is dependent on CD8+ lymphocytes while CD4+ cell

participation is required for rejection of grafts expressing only class II differences (Rosenberg et al., 1986,1987). These observations are compatible with the theory that the T cell, and more specifically TCR recognition of foreign MHC, is responsible for rejecting grafts.

T Lymphocyte Activation

In a primary immune response, T cell activation occurs when the antigen specific TCR is stimulated by a foreign antigen in the context of an appropriate MHC antigen. Following activation, the cell undergoes dramatic changes including an increase in cell volume (blast transformation), an increase in DNA synthesis and the generation of specific cell products. These events result in clonal expansion of specific T cells. Additionally, the cells differentiate and acquire specific functions associated with T helper or T cytotoxic/suppressor cells. T helper cells (CD4+) produce interleukin-2 (IL-2) which serves as the necessary secondary signal (following antigen stimulation) for inducing proliferation of T cells (Bonnard et al., 1979; Smith, 1980) as well as for generating CTL activity (Farrar et al., 1981). The ability of T cells to respond to IL-2 depends on activation of the cells to express the high affinity IL-2 receptor (IL-2R) (Bonnard et al., 1979; Smith, 1980).

The IL-2 Receptor

The IL-2R has been identified and described as a dimer consisting of a p55 alpha chain with low affinity for IL-2 and a p75 beta chain with slightly greater IL-2 affinity (Greene et al., 1986; Tsudo et al., 1986; Teshigawara et al., 1987; Smith, 1988a). With mitogenic or allogeneic stimulation, the two chains are expressed

together on the cell surface to form the high affinity IL-2R complex which, when bound to IL-2, is largely responsible for signaling cell proliferation (Sharon et al., 1986; Smith, 1988b). The significance of this high-affinity IL-2R has been demonstrated by experiments in which an antibody to the IL-2R (anti-Tac) blocks IL-2 binding (Robb et al., 1981; Leonard et al., 1982; Sharon et al., 1986), thereby inhibiting cell proliferation (Robb et al., 1981). In addition, antibody blocking of the IL-2/IL-2R interaction has effectively prevented allograft rejection (Kirkman et al., 1985). While T helper cells serve as the source of IL-2, IL-2R are expressed on T helper, T cytotoxic/suppressor and even activated B cells (Waldmann et al., 1984). Thus, IL-2 can have a significant impact on several cell types involved in cell-mediated immune responses.

Cytotoxic T Lymphocytes

Cytotoxic T cell differentiation and activation are dependent on IL-2 production by T helper cells (Nabholz and MacDonald, 1983). This effect of IL-2 is largely responsible for the synergism of the two cell types that has been observed both in vivo (Cantor and Asofsky, 1971) and in vitro (Wagner, 1973). While IL-2 has been promoted as the lymphokine responsible for the secondary stimulus required for complete CTL activation, it has recently been reported that IL-4 may be equally effective in its ability to serve as a helper factor for CTL generation (Widmer and Grabstein, 1987). Nevertheless, IL-2 induction of CTL proliferation may be an important event associated with graft rejection. Convincing evidence exists for CTL involvement in the rejection process. For example, using flow cytometric analysis, a shift in the ratio of CD4+ to CD8+ cells (from 1.6 on day

3 to .7 on day 5), was found to be associated with the period of most intense immunological graft destruction (Tilney et al., 1986). In addition, specific, MHC-restricted allograft rejection in vivo by cloned CTLs has been reported (Snider et al., 1986).

Additional Cell Types Affected by IL-2

In addition to its effects on T cells, IL-2 can promote the activation of other cells associated with, or involved in, the rejection of allografts. Activated B cells express the high affinity IL-2R (Waldman et al., 1984) and IL-2 promotes both proliferation and differentiation of B cells. Furthermore, IL-2 regulates B cell production of antibodies (Jelinek and Lipsky, 1987). At high concentrations, IL-2 has been shown to enhance cytotoxic activity in macrophages (Malkovsky et al., 1987). Similarly, NK cells respond to IL-2 with augmented cytotoxic activity, IL-2 receptor expression, proliferation and increased interferon gamma production (Trinchieri et al., 1984; Brooks and Henney, 1985; Malkovsky et al., 1987). Together, these findings indicate multiple regulatory effects of IL-2 on immune function.

A role for NK cells and/or macrophages in graft rejection has not been elucidated. While they do exist as somewhat minor populations in cellular infiltrate of rejected grafts (Hayry et al., 1984; Tilney et al., 1984), their significance is not understood. Macrophages participate in cell mediated immunity by interacting specifically with lymphocytes to promote antigen-specific responses, by their ability to phagocytize cells specifically labeled by antibodies and by direct cytotoxicity (Anderson and Hill, 1988).

Similarly, NK and killer cells are capable of cytolytic activity mediated by antibodies.

Suppressor Cells

While attempts to maintain allografts have focused mainly on procedures that might interfere with or reduce effector cell function, an alternate method might be through the induction of immune suppressor cells. Results of various methods used to identify suppressor cells have implicated both T and B lymphocytes (Katz et al., 1974; Zubler et al., 1980) as well as macrophages (Baird and Kaplan, 1977; Oehler et al., 1977) as mediators of suppression. However, overwhelming evidence indicates that T cells, specifically CD8+ cells, are responsible for most suppressor cell activity. When CD8+ cells were added to mixtures of B cells and T helper cells, antibody production was substantially reduced (Hodes, 1989). It has been suggested that suppressor cells serve more as homeostatic regulators of immune responses than mediators of complete unresponsiveness (Eardley and Gershon, 1975; Eardley et al., 1978). Such regulatory mechanisms could be associated with allograft acceptance and/or rejection. Antigen non-specific T suppressor cells have been identified during graft-versus-host disease following bone marrow transplantation (Pickel and Hoffmann, 1977; Shearer and Polisson, 1980), indicating a role for these cells in failed grafts. There is also evidence for suppressor cell involvement in the fetal allograft phenomenon and these studies will be discussed in the following sections. Unfortunately, even with the tremendous quantity of information pertaining to suppressor cells, the basic mechanism underlying the functions of such cells remains largely unresolved.

In Vitro Assessment of Immune Cell Function

Mitogen-stimulated Proliferation

While lymphocyte activation generally requires stimulation by antigen in the context of MHC, there are reagents available that obviate the need for specific antigen stimulation. These reagents include lectins or mitogens which can polyclonally activate cells by binding non-specific carbohydrate groups on the lymphocyte surface (Sharon, 1983). Phytohemagglutinin (PHA) (Nowell, 1960), concanavilin A (Con A) and pokeweed mitogen (PWM) were among the first lectins identified. The ability of Con A and PHA to selectively stimulate T cells is likely due to their binding of carbohydrate groups expressed on T cell receptors (Sharon, 1983). In fact, results from recent studies indicate the lectins bind directly to components of the T cell receptor (Weiss et al., 1987). In contrast to these T cell mitogens, PWM primarily stimulates B cells while also affecting T cells. Lipopolysaccharide (LPS) endotoxin also serves effectively as a mitogen, but it non-specifically stimulates B cells to proliferate, differentiate and produce antibodies (Kincade and Gimble, 1989). These potent lymphocyte stimulators have facilitated the study of non-specific immune cell responses in vitro.

Mixed Lymphocyte Reaction

Another in vitro assay that has been extensively used to investigate cell mediated immune responses is the mixed-lymphocyte reaction (MLR). This assay exploits the fact that T cells recognize non-self MHC antigens as foreign, and the stimulation by this "foreign" antigen causes the cells to undergo transformation and proliferation. Because of this blast response by lymphocytes to

allogeneic stimuli, the assay has been referred to as the in vitro correlate of allograft rejection (Andersson and Hayry, 1974). Cell proliferation in this assay can be assessed by measuring cell uptake of a radiolabeled precursor supplemented to the culture medium. Due to the nature of the cellular response in this assay, it has been used for tissue typing (Roit, 1987) and is still frequently used to assess the relatedness of two individuals. In addition, MLRs have been used to identify suppressor activity of immune cells obtained from the placenta (Uren and Boyle, 1990) and to assess soluble suppressor factors found in conceptus culture medium (Murray et al., 1987).

Cytolytic Cell Assays

The ability to measure cytolytic activity of cytotoxic T cells by using target cells that have incorporated ^{51}Cr, released and measurable upon lysis of that cell, provides another mechanism that can be used when examining antigen-specific, cell-mediated immune responses in vitro. Such an assay is particularly relevant in studies of allograft rejection because of the involvement of CTL in this response. In contrast to the cytolytic assay, a CTL proliferation assay is used to quantify IL-2. Due to the potent effect of IL-2 on CTL proliferation, it was possible to establish a murine cytolytic T lymphocyte line (CTLL) for measuring IL-2. These CTLL cells constitutively express the high affinity IL-2R and proliferate only in response to IL-2. In the absence of IL-2, the cells cease to proliferate and die within 12-18 hours. Even more important, the degree of CTLL proliferation is dependent on the concentration of IL-2. Therefore, quantitative measurements of IL-2 activity in various samples can be determined with these cells (Gillis et al., 1978).

THE PUTATIVE IMMUNO-PROTECTIVE ROLE OF THE PLACENTA

The Phenomenon of the Fetal Allograft

Mammalian pregnancies are generally characterized by the successful development of allogeneic conceptuses intimately associated with maternal uterine tissue. Maternal acceptance of the fetal allograft represents a unique case of immunological tolerance that is consistently reproducible. For decades scientists have been challenged to explain how the feto-placental unit survives to term while artifactual allografts repeatedly fail.

In 1953, Medawar offered potential solutions to the mystery. Based on his solutions, Billingham (1964) and Simmons (1969) formulated hypotheses pertaining to the mechanisms possibly involved. The proposed mechanisms included: 1) The uterus is an immunologically privileged site 2) The conceptus is antigenically immature 3) The placenta provides a barrier to cellular and humoral maternal-fetal interactions 4) The maternal immune system is suppressed and 5) The immune response of the mother is qualitatively different during pregnancy such that it promotes fetal survival. The first option has been eliminated (Beer and Billingham, 1976) since it has been shown that the uterus possesses an immune system capable of tissue-graft rejection (Reimers and Dzuik, 1974). There is substantial evidence against the second option as the conceptus expresses several classes of alloantigens and isoantigens on its cell surfaces (Wegmann et al., 1979; Chatterjee-Hasrouni and LaLa, 1982; Lala et al., 1983; Johnson, 1984; Crump et al., 1987; Donaldson et al., 1990). Furthermore, the presence of anti-fetal antibodies frequently identified in maternal circulation, refute both options 2 and 4 (Terasaki et al., 1970;

Newman and Hines, 1980; Smith et al., 1982; Antczak et al., 1984). Finally, there is a complete lack of evidence supporting option five. The potential role of the placenta as the primary barrier to immunological rejection (option 3) has increasingly received greater attention with the accumulation of evidence against the other hypotheses.

The Equine Placenta

The term "placental barrier" has long been used to describe the tissue layers separating fetal and maternal blood supplies. The role of the placenta as a "barrier" differs both structurally and physiologically between species. Equine placentae, in particular, are characterized by specialized structures with functions unique to the species. Because the research described in this dissertation focuses largely on the horse conceptus, a brief description of implantation and placental formation in this species is warranted.

Oviductal transport in the mare appears to be selective for fertilized embryos, and oocytes are only rarely found in the uterus. Horse embryos enter the uterus as early blastocysts on day 5 or 6 after ovulation (Hamilton and Day , 1945; Oguri and Tsutsumi, 1972). Normally developing embryos hatch from their zonae pelucidae on day 8 or 9, but remain encapsulated by another non-cellular, translucent membrane referred to as the embryonic capsule (Flood and Betteridge, 1982). Trans-uterine migration of the embryonic vesicle ceases by day 15 when fixation occurs in one of the uterine horns (Ginther, 1986). Vesicle expansion and fetal development proceed, but actual implantation does not begin until day 36 of gestation (Allen et al., 1973).

Placentation in equids is uniquely characterized by the formation of structures known as endometrial cups. These cups originate from specialized trophoblast cells, chorionic girdle cells, which invade the maternal endometrium between days 36 and 38 after ovulation (Allen and Moor, 1972; Allen et al., 1973). Within 24 hours, these cells migrate into the uterine stroma, increase in size and begin secreting equine chorionic gonadotrophin (eCG). Leukocytes, primarily lymphocytes, immediately accumulate around the developing endometrial cups and their numbers increase as gestation progresses (Allen, 1979). While these cells appear to "wall off" each cup from the uterine stroma, it is not until day 70 or 80 that the lymphocytes invade the cups. This invasion of immune cells leads to the destruction of the endometrial cups and by day 120, the tissue sloughs into the uterine lumen.

The nature of the intense lymphocyte accumulation surrounding the endometrial cups remains unclear. A similar response is not observed around the non-invasive trophoblast of the allantochorion (Allen, 1975) indicating that cup cells may express antigens not present on other trophoblast cells. The fact that antibodies to fetal major histocompatibility complex (MHC) antigens are present in 90% of mares carrying MHC-incompatible fetuses (Bright et al., 1978; DeWeck et al., 1978), and that these antibodies are detected 2 to 4 weeks after cup formation (Antczak et al., 1984) would indicate that paternal MHC-antigens are present on endometrial cup cells. Indeed, Crump et al. (1987), provided conclusive evidence for the expression of paternal MHC antigens on the invasive chorionic girdle cells at day 33 of gestation. In addition, Donaldson et al. (1990), found that

class I MHC antigen expression was largely restricted to the early stages of trophoblast invasion. They reported a reduction in MHC antigens on mature cup cells and suggested that developmentally regulated MHC expression may serve as one defense against alloimmune rejection mechanisms. Surely, this phenomenon supports the suggestion that the placenta plays a role as an immuno-protective barrier for the fetal allograft. Conversely, a vigorous leukocyte reaction against MHC-compatible endometrial cups has been observed (Allen et al., 1983; Antczak and Allen, 1984) indicating that MHC antigens are not primarily responsible for the immune response against endometrial cups.

Hybrid and Interspecific Pregnancies

The study of both successful and failed hybrid and interspecific pregnancies, has led to a greater appreciation for the placenta's role in preventing immunological rejection of the fetus. The three most thoroughly studied models of hybridism are the murine model of *Mus musculus* and *M. caroli*, the equine model involving primarily the domestic horse and donkey and a model involving two genera, the domestic sheep (*Ovis aries*) and goat (*Capra hircus*). While exhibiting some similarities, each model is unique with respect to success rates, complications and characteristic abnormalities relative to intraspecific pregnancies. Immunological interference appears to be involved in, if not responsible for, anomalies associated with interspecific pregnancies, but how this immune interference is manifested differs with species.

The Murine Model

The murine model of interspecific pregnancy has been intensively studied (Rossant et al., 1982; 1983a and Croy, 1985). While *M. caroli* and *M. musculus* have the same diploid chromosome number they do not readily hybridize. Both fertilization and postimplantation survival rates are extremely low (West et al., 1977), but such failure likely results from retarded development of hybrids relative to either parental species (Frels et al., 1980). Such retarded development indicates genomic incompatibility and not immunological interference as the primary cause of pregnancy failure, and will therefore not be discussed further. Results from interspecific embryo transfer between the two murine species have revealed a more puzzling situation. Survival to term of *M. caroli* embryos transferred to *M. musculus* recipients was very low (1/69) while M. musculus embryos transferred to the same recipients developed normally (Frels et al., 1980). Furthermore, Croy et al. (1982) noted that after 9.5 days of apparently normal development, the *M. caroli* placental trophoblast layer became infiltrated with lymphocytes and hemorrhage occurred, while *M. musculus* embryos in the same uterus were unaffected. Cytotoxic T lymphocytes that lysed *M. caroli* target cells were identified at the resorption sites indicating immunological involvement in the demise of *M. caroli* embryos. Additional evidence for immunological involvement in fetal survival was reported by Clark et al. (1983) who found that while decidual cells with immunosuppressive capabilities are present during intraspecies pregnancy in one uterine horn, they are not detectable in decidua of xenogeneic (*M. caroli*) conceptuses that have implanted in the opposite

uterine horn. It was hypothesized that *M. caroli* trophoblast failed to recruit or stimulate these suppressor cells.

The fact that the conceptus represents a unique type of allograft is supported by the results of experiments involving the transfer of *M. caroli* embryos to *M. musculus* recipients with genetically or experimentally altered immune function. *M. caroli* survival in *M. musculus* females was not improved when recipients were treated with Cyclosporin A which is capable of inhibiting T-lymphocyte mediated rejection of transplants (Croy et al., 1985b). In the same study, *M. musculus* recipients that were either athymic (T cell deficient) or natural killer (NK) cell deficient failed to support *M. caroli* embryo development, yet the athymic recipients did accept *M. caroli* skin grafts. While these data may refute the hypothesis of immune system involvement with interspecific pregnancy failure in this model, the results are complicated by the confounding effect of immune system alterations on pregnancy in general. Difficulties were reported maintaining pregnancies in athymic M. musculus females following the transfer of *M. musculus* embryos (Croy et al., 1985b). The failure of putatively immuno-incompetent *M. musculus* mice to support *M. caroli* embryos has been explained as a result of *M. musculus* hypersensitivity (pre-existing immunity) to *M. caroli* antigens (Clark et al., 1986). However, the basis for this pre-existing sensitivity was not given.

Why interspecific pregnancies proceed successfully when *M. musculus* embryos are transferred to *M. caroli* recipients, yet fail consistently when the species of embryo and recipient are reversed, remains unclear. It has been reported that fewer suppressor cells are

present in the decidua of *M. caroli* conceptuses than *M. musculus* conceptuses during intraspecific pregnancies (Clark et al., 1986). In addition, *M. caroli* placental cells exhibited less inherent ability to inhibit cytotoxic effector cell killing when compared to *M. musculus* placental cells. In view of this data, it has been suggested that, compared to *M. musculus*, *M. caroli* conceptuses have a weakened defense against maternal immune attack even in the *M. caroli* uterus (Anderson, 1988). Even with the substantial quantity of information obtained, the exact mechanism responsible for the failure or success of *M. musculus/M. caroli* interspecific pregnancies has not been defined. However, it is widely believed that a maternal immune response and a failure of conceptus-derived local immunoregulation are primary contributors to the death of xenogeneic embryos.

The Sheep/Goat Model

Scientists have been puzzled for years over the failure of domestic sheep x goat hybrid pregnancies when the two species are frequently observed mating. Interspecific fertilization failure was initially suggested as an explanation for failure of hybrid pregnancies, but this theory was refuted long ago (Warwick and Berry, 1949; Bratanov and Dikov, 1962). Despite the fact that fertilization between sheep and goats does occur, hybrid embryos invariably die during the first 2-3 months of gestation (Warwick and Berry, 1949; Alexander et al., 1967). Death does not appear to be caused by cytological anomalies associated with altered chromosome numbers (Buttle and Hancock, 1966). In addition, Hancock and Jacobs (1966) found no evidence of developmental abnormalities that might explain early death. It was suggested that because the caprine placenta

differs from that of the ovine in that it is not a source of progesterone (Amoroso and Perry, 1975), the hybrid placenta may prove to be deficient in progesterone production. Such a hormone deficiency could lead to endocrine-associated pregnancy failure. However, McGovern (1976) was unsuccessful in extending the survival of the hybrid fetus by administering exogenous progesterone to recipient does.

While all of the factors mentioned above may contribute to sheep x goat hybrid pregnancy loss, there is an overwhelming body of evidence which suggests that pregnancy failure is due to immunological incompatibility between maternal and fetal tissues. Numerous reports of abnormal histological and ultrastructural characteristics of placentae in hybrid pregnancies (Alexander et al., 1967; Hancock et al., 1968; McGovern 1973a; Dent et al., 1971a,b) support the idea that the maternal immune reaction is directed against the hybrid placenta and not the fetus itself. Hybrid placentae development was extensively studied by Hancock et al., 1968. They reported that in some pregnancies the fetal membranes failed to establish normal contact with the uterus and caruncular development was incomplete. A similar deficiency in trophoblast invasion has been reported following the transfer of sheep embryos to goat recipients (Hancock et al., 1968). In either case, fetal death results. By contrast, in other hybrid pregnancies and when goat embryos developed in sheep recipients, cotyledonary development was prominent and invasion of uterine tissue by trophoblast was extensive. In such cases, necrosis of both fetal and maternal tissues occurred, accompanied by maternal immune cell infiltration. Large aggregation of platelets and lobular

swelling of the endothelium were identified in maternal capillaries beneath the uterine epithelium. Similar observations are characteristic of viscera undergoing rejection and of blood vessels damaged by antigen-antibody complexes. The fact that hybrid pregnancy failure occurs earlier during gestation in second-time recipients or in recipients immunized with xenogeneic paternal leukocytes, supports the theory that an immunological reaction is the primary factor resposible for hybrid fetal death (McGovern, 1973).

The Equine Model

Equidae are unique in that hybrid and interspecific pregnancies are possible across a variety of species with wide chromosomal differences (Short et al., 1974; Benirshke and Ryder, 1985). Nevertheless, relative to intraspecific pregnancies, there are significant alterations in endometrial cup formation and survival, eCG secretion and leukocyte accumulation in interspecific or hybrid pregnancies. Results from numerous studies indicate that the immune system is primarily responsible for aberrant cup formation.

The transfer of horse embryos to donkey recipients appears to be more successful than the transfer of donkey embryos to horses. Compared to intraspecific donkey pregnancies, horse-in-donkey pregnancies result in the formation of larger endometrial cups surrounded by excessive numbers of accumulated leukocytes (Allen et al., 1985). Despite the increased number of leukocytes, the cups remain intact past day 100 of gestation and pregnancy proceeds successfully. In contrast, donkey embryos develop normally in horse recipients up to day 70 despite a complete lack of endometrial cup formation. Thereafter, they succumb to a vigorous cell-mediated

maternal immune response directed against the allantochorion. Similar to observations of horse-in-donkey pregnancies, are reports of hinny pregnancies (female donkey bred to male horse) in which there are an excessive number of leukocytes surrounding the highly invasive endometrial cup tissue yet the cups maintain a normal lifespan of 80 to 100 days (Allen, 1982b). In contrast, mule pregnancies (female horse bred to male donkey) are characterized by intensive lymphocyte infiltration deep into the endometrial cups which results in complete loss of cup tissue by day 60 of gestation (Allen et al., 1982b). Surprisingly, mule pregnancies are successful despite the early loss of endometrial cup tissue.

Various therapies have been used in hopes of promoting the successful maintenance of donkey-in-horse pregnancies. Daily administration of a synthetic progestin to mares with donkey fetuses failed to prevent pregnancy failure (Allen 1982b; Allen et al., 1985). Injections of partially purified eCG obtained from mares carrying intraspecific pregnancies also proved to be mostly ineffective in prolonging donkey pregnancies in mares. However, a dramatic improvement in such pregnancies was observed when recipient mares were immunized with parental donkey lymphocytes (Antczak and Allen, 1984). Allen et al. (1987) went on to find that such an immunization regimen was successful even if the lymphocytes were not of parental origin. Based on the results of donkey-in horse pregnancies, it has been proposed (Antczak and Allen, 1984; Allen et al., 1985) that antigens on the invading chorionic girdle cells stimulate a maternal immunoprotective response and if cup formation fails, the response remains unsolicited and immune rejection proceeds.

Exotic Interspecies Pregnancies

The phenomenon of interspecific and hybrid pregnancy is not limited to domestic species. As stated previously, there are a number of viable equine hybrids. One example is the Przewalski (*Equus przewalski*, 2n=66) x domestic horse (*E. caballus*, 2n=64) hybrid. The success of this cross led to embryo transfer attempts from endangered przewalskis to domestic mares (Hearn and Summers, 1986; Summers et al., 1987). Many of the established interspecific pregnancies developed to term and the presence of cytotoxic antibodies to parental antigens, as well as normal eCG levels in maternal sera, indicated normal endometrial cup formation. Perhaps more surprising is the successful transfer of Grant's zebra (*E. burchelli*, 2n=44) embryos to domestic horses (Bennet and Foster, 1985; Kydd et al., 1985; Hearn and Summers et al., 1986; Summers et al., 1987). Transfer of Grant zebra embryos to donkey recipients was also attempted (Kydd et al., 1985; Hearn and Summers, 1986; Summers et al., 1987) and pregnancies were established, but no live young were born. One animal aborted her fetus at day 292 of pregnancy and exhibited symptoms of immunologically based pregnancy toxemia syndrome. Pregnancy failure in the other recipients was characterized by very low levels of eCG produced for only a brief period of time suggesting poor endometrial cup formation and a strong cell-mediated cytotoxic reaction.

Among Bovidae there have been viable hybrid offspring produced, one example being the Zebu cattle (*Bos indicus*) x European domestic cattle (*B. taurus*) cross (Summers et al., 1983b). In addition, the American bison (*Bison bison*) will hybridize with European bison (*Bison. bonasus*), *B. indicus*, *B. taurus* and the Yak (*Bos grunniens*).

Bovidae interspecific and intergeneric pregnancies established following embryo transfer have yielded variable results. A pregnancy resulting from the transfer of a *B. taurus* embryo to a water buffalo (*Bubalus bubalus*) was aborted by 3 months of gestation and no pregnancies resulted from reciprocal transfers (Drost et al., 1986). Stover et al. (1981) reported the birth of a viable gaur calf (*Bos gaurus*) to a domestic cow recipient (*B. taurus*) following embryo transfer, however the other four recipients lost their pregnancies prior to term. In addition, the authors of this study reported abnormally low numbers of cotyledons on the allantochorion of both successful and aborted pregnancies indicating inappropriate placental-uterine tissue interaction. In contrast, reports of failed Dall's sheep (*Ovis dalli dalli*) pregnancies established following embryo transfer to domestic sheep (*O. aries*) indicated that there were no significant differences in cotyledon numbers (Buckrell et al., 1990). As there does not appear to be a specific characteristic associated with interspecific pregnancy failure, it has been difficult to predict and/or explain the success or failure of such pregnancies. However in most, if not all cases of interspecific pregnancy failure, immunological involvement is implicated. Nevertheless, the few successful exotic-in-domestic species pregnancies as well as the successful transfer of embryos between two exotic species such as the bongo antelope (*Tragelaphus euryceros*) to African eland (*T. oryx*) (Dresser et al., 1985) maintain interspecific embryo transfer as a potentially viable option for facilitating the propagation of endangered species.

Embryo Manipulation

Perhaps the most convincing evidence supporting the hypothesis that the placenta is responsible for fetal allograft survival has been obtained from studies involving pre-implantation embryo manipulation. As embryos develop from morulae to blastocysts, the first irreversible cell differentiation occurs and two distinct cell lineages emerge, the inner-cell-mass (ICM) and the trophectoderm. The ICM cells continue to differentiate and become specialized, eventually completing the formation of the fetus. The trophectoderm, or trophoblast cells, possess the inherent ability to initiate implantation (Gardner et al., 1973) and placental tissue is derived almost entirely from these cells.

In 1961, Tarkowski first reported that viable offspring could be produced from composite embryos derived from two aggregated eight-cell stage mouse embryos. Such offspring, containing cell populations of more than one genotype, are commonly referred to as chimeras. Over the past several years, a great deal of progress has been made concerning embryo manipulation. Methods for producing chimeras, other than by embryo aggregation, were identified. For example, the blastocyst injection technique was described by Gardner et al. (1973) and later simplified by Butler et al. (1987). This procedure involves the injection of an ICM isolated from one blastocyst into a recipient blastocyst. The end result is generally a chimeric fetus surrounded by non-chimeric trophoblast.

Using blastocyst injection, Rossant and Frels (1980) injected *M. caroli* ICMs into *M. musculus* blastocysts and healthy, viable chimeric offspring were produced in *M. musculus* recipients. As this procedure

ensured that the trophoblast, and thus the placenta, was derived from *M. musculus* cells, the recipients were prevented from immunologically rejecting the *M. caroli* cells contributing to the fetus proper. The importance of the trophoblast in maintaining interspecific murine pregnancies was further clarified when Rossant et al. (1982b) reported that the injection of *M. musculus* ICM into *M. caroli* blastocysts did not result in viable chimeric offspring if these composite embryos were transferred to *M. musculus* recipients. In addition, Papaioannou (1981) used a procedure known as blastocyst reconstitution, whereby the ICM of one blastocyst is completely replaced by the ICM of another, to produce a *M. caroli* offspring surrounded by *M. musculus* trophoblast carried to term in a *M. musculus* recipient. For fetal allograft survival, it appears to be imperative that the trophoblast and recipient are of the same species.

The success of chimeric and interspecific pregnancies following embryo manipulation is not restricted to the murine species. Such procedures have been applied to pre-implantation sheep and goat embryos over the past several years with results similar to those reported for mice. A number of scientists have produced interspecific sheep <-> goat chimeras by aggregating cells from both sheep and goat embryos (Fehilly et al., 1984) and by blastocyst injection (Fehilly et al., 1984; Polzin et al., 1986, 1987; Roth et al., 1989). In most cases, the manipulations were carried out in a manner that favored the development of trophoblast from cells of the same species as the recipient. However, it is worth noting that in cases of failed chimeric pregnancies, interspecific chimeric trophoblast was suspected

and placental formation was characterized by fewer placentomes (Anderson, 1988).

Blastomere aggregation and ICM injection are two techniques that have been used successfully in promoting true interspecific pregnancies. By combining blastomeres from embryos at slightly different stages of development, such that the ICM is derived mainly from one embryo and the trophoblast from the other, both goat-in-sheep (Meinecke-Tillman and Meinecke, 1984) and sheep-in-goat (Fehilly and Willadsen, 1986) pregnancies have been carried to term. Similarly, interspecific injections of sheep ICM into goat blastocysts or goat ICM into sheep blastocysts have occasionally resulted in non-chimeric lambs born to goats and kids born to sheep (Fehilly et al., 1984; Polzin et al., 1987). Furthermore, the injection of sheep x goat hybrid ICM into sheep blastocysts followed by their transfer to sheep recipients has resulted in sheep x goat hybrid <-> sheep chimeras (Roth et al., 1989). These results indicate that sheep x goat hybrid cells are viable and can contribute to the fetus and that hybrid pregnancy loss likely results from improper uterine-placental interactions, not aberrant fetal development.

While the significance of the placenta in protecting the fetal allograft is becoming well-established, the mechanism through which protection is mediated remains unclear. The theory that the placenta "masks" foreign fetal antigens from maternal immune recognition is flawed in that antibodies to fetal antigens are detected in the circulation of pregnant females. More specifically, antibodies to both allogeneic and xenogeneic antigens have been identified in sheep and goats carrying interspecific, chimeric pregnancies (Ruffing et

al., 1988). Even when ICM injection techniques are used such that non-chimeric trophoblast is ensured, the mother recognizes and responds, at least via humoral immunity, to xenogeneic fetal antigens (MacLaren et al., 1990).

Pertinent information concerning the immuno-protective role of the placenta has been obtained from studies of hybrid and interspecific pregnancies as well as from experiments involving embryo manipulation. Despite the progress that has been made, there is no definite single answer to the proposed question concerning fetal allograft survival. Most likely, the mechanism responsible is complex, involving specific placental, uterine and immune system interactions that will require years of study to fully understand.

IMMUNOSUPPRESSION AND PREGNANCY

Antibody Involvement

Following the establishment of immunocompetency in pregnant females and the detection of maternal immune responses generated against fetal antigens, stronger interest was expressed in the theory that an immunosuppressive mechanism localized at the feto-maternal interface is responsible for fetal allograft survival. The presence of anti-fetal antibodies in serum of pregnant females has been discussed. The capacity of the placenta to act as an antigen-expressing "sponge" for absorbing these antibodies has been suggested (Raghupathy et al., 1981; Raghupathy et al., 1984), but Bell and Billington (1983) found that the "sponge" activity of the placenta was ineffective when serum contained higher antibody titers. Alternatively, it has been suggested that anti-paternal antibodies

which accumulate in and around the placenta (Hunziker et al., 1986) act as blocking antibodies. Such antibodies obtained from parous serum (Pavia and Stites, 1981; Hellstrom and Hellstrom, 1975) or eluted directly from the placenta (Bonneau et al., 1973; Faulk et al., 1974; Stewart et al., 1984) are capable of suppressing lymphokine secretion, mixed lymphocyte reactions, and the generation of cytotoxic killer cells in vitro (Rocklin et al., 1979). However, the relevance of these blocking antibodies is questionable in light of the fact that not all successful pregnancies are associated with a humoral immune response (Lala et al., 1983) and thus, perhaps more important for fetal allograft survival, is a local suppression of cell-mediated immunity.

Suppressor Cells

Localized down-regulation of maternal cell-mediated immune responses could occur via recruitment/activation of suppressor cells or by the production of soluble immunosuppressive factors. Supporting evidence for the former has been reported by Clark and colleagues who have identified suppressor cells in uterine draining lymph nodes and decidual cells scraped from the gravid uterus (Clark et al., 1980). Two different populations have been identified as phase A and phase B cells (Slapsys and Clark, 1983). The phase A cells are produced in response to hormonal changes and prevent the generation of cytotoxic T lymphocytes (CTLs), but are only present during the preimplantation phase of gestation (Clark et al., 1980; Brierley and Clark, 1985). Phase B suppressor cells are described as non-T, non-B lymphocytes produced only when viable trophoblast is present. The absence of these cells is associated with failed *Mus musculus* x *M. caroli* hybrid

pregnancies (Clark et al., 1980; Clark et al., 1983). Phase B cells express antigen-non-specific suppressor activity which inhibits T cell activation in response to IL-2 (Clark et al., 1985). While these two types of suppressor cells may play an important role at the feto-maternal interface, their activity can be mediated by a soluble factor of the decidua (Clark et al., 1984), indicating that the suppression of cell-mediated immunity in the uterus may ultimately be attributed to soluble factors produced by the trophoblast.

Macrophage Involvement in Immunosuppression

While many investigations have focused on lymphocytes as the target cell type for soluble immunosuppressors derived from the conceptus, there is some evidence that macrophages also play a role in suppressing immune reactivity in and around the placenta. Uren and Boyle (1990) recently reported that macrophages isolated from human placentae elicited only a low proliferative response from allogeneic T cells and failed to incite CTL function. The suppressive effect of these macrophages was not inhibited by indomethacin, indicating the activity was independent of prostaglandin production. These results differ from those reported by Scodras et al. (1990) who found that, in the mouse, decidual macrophages inactivated NK cells by their production of PGE_2.

Suppressor Activity of Prostaglandins and Hormones

It has been well established that prostaglandins, especially PGE_2, are effective immunosuppressors. PGE_2 is a feedback inhibiter of cellular immune functions and is produced by suppressor cells (Goodwin and Ceuppens, 1985). Although PGE_2 is produced in culture by embryos of numerous species including humans (Holmes et al., 1990),

cows (Hwang et al., 1988), sheep (Lacroix and Kann, 1984) and rabbits (Pakrasi and Dey, 1982), its role at the feto-maternal interface as a product of conceptus tissue is not well defined.

More attention has been focused on the potential significance of conceptus-derived, hormone-mediated suppression of immune function. Specifically, two steroid hormones, progesterone and estrogen, which play a critical role in the maintenance of pregnancy and are produced by the conceptus (Heap et al., 1982; Dickmann et al., 1976; Flood et al., 1979) have been studied for their immunoregulatory capacities.

In 1953, Black et al. first reported a decreased resistance to infection in the pseudopregnant rabbit uterus as compared to that of the estrus rabbit. However, it was many years later that truly compelling evidence for progesterone as a uterine immunosuppressant was reported. Moriyama and Sugawa (1972) demonstrated that, following progesterone treatment, xenogeneic cell survival in the hamster uterus was prolonged. Similarly, Siiteri et al. (1977) implanted steroid containing silastic implants into murine uteri and found that such treatment resulted in a local suppression of immunity and prolongation of xenogeneic skin grafts. In addition to these in vivo investigations, numerous in vitro studies have been carried out to characterize the immunoregulatory activity of progesterone. In general, the results indicate that progesterone inhibits mitogen activation of T lymphocytes when present in concentrations ranging from 10-20 μM or greater (Shiff et al., 1975; Mendelsohn et al., 1977; Mori et al., 1977; Clemens et al., 1979), it inhibits T cell response to allogeneic cells in a MLR (Clemens et al., 1979) and can block the generation of CTLs directed against MHC antigens (Pavia and Stites,

1981) when present at 1 to 3 µg/ml. However, progesterone can not affect CTL activity once the cells are produced, and progesterone is lethal to cells at 20 µg/ml (Siiteri and Stites, 1982). The significance of progesterone as a local immunosuppressor in utero is questionable considering only picogram-nanogram concentrations of hormones are secreted by the conceptus in culture or found in blastocoelic fluid of embryos (Flood et al., 1979; Marsan et al., 1987).

Estrogen has received much less attention as an immunoregulatory factor, partly because its effect on immune cell function is contingent on several factors. Estrogen can act as either a suppressor or an enhancer of immune function depending on characteristics of the host, such as age (Reilly et al., 1967), genome (Stern and Davidsohn, 1955), species (Nelson et al., 1967) and timing of immunization (Feigen et al., 1978). While results from in vitro studies with human lymphocytes have demonstrated the capacity of estrogen to suppress mitogen, antigen or allogeneic cell activation, as with progesterone, the levels of estrogen required were far above physiological concentration (Mendelsohn et al., 1977; Neifeld and Tormey, 1979).

Soluble Suppressor Factors

Murine

The soluble placental factor responsible for inducing suppressor T cells during murine pregnancies was first partially characterized by Dutchet-Suchaux et al. (1979). In 1984, Chaouat and Chaffaux reported that this factor, known as the Suppressor Inducer Factor (SIF), could induce suppressor cells in the Graft-vs-host reaction.

Similar suppressor activities have been identified in supernatants of cultured murine blastocysts (Mayumi et al., 1985). Furthermore, it has been shown that supernatants from murine decidua can block mixed lymphocyte reactions (Chaouat and Kolb, 1984) and have effectively enhanced the survival of a murine heart allograft (Chaouat, 1987).

In addition to SIF, other immune modulatory factors associated with murine pregnancy have been identified. The resistance of placental tissue to lysis by natural killer cells and cytotoxic T lymphocytes is associated with the production of two distinct molecules that inhibit NK and CTL activity (Chaouat et al., 1985). There is evidence that NK cell infiltration into the decidua is associated with spontaneous abortion following matings of CBA female mice with DBA/2J males (Gendron and Baines, 1988). These findings may indicate a requirement for inhibiting NK cell activity to maintain successful pregnancies.

While placental-derived suppressor factors may be ultimately responsible for locally inhibiting the immune response in the uterus, they may be acting indirectly through the activation of suppressor cells responsible for suppression of cell-mediated immunity. The suppressor cells obtained from murine decidua secrete a 100,000 dalton factor that, in turn, blocks the response of T cells to IL-2 (Clark et al., 1985; Clark et al., 1986). In summary, murine decidua and/or conceptuses produce soluble factors that regulate immune suppressor cells. These cells produce soluble factors capable of interfering with cell-mediated immune responses.

Human

While the murine species has been used most extensively in studies of placenta-derived, immunoregulatory molecules, a substantial quantity of information is available concerning similar factors in the human. Decidual cells obtained from endometrium of first trimester human pregnancies suppressed lymphocyte responses when supplemented to MLR cultures (Nakayama et al., 1985). In addition, the production of immunosuppressor factors by pre-implantation embryos fertilized in vitro has been reported (Daya and Clark, 1986). These authors suggested that the detected production of suppressor factors from only 43% of the embryos could, in part, explain the high percent of embryos lost very early in pregnancy.

Two suppressor factors associated with pregnancy in humans have received considerable attention. A specific factor known as placental protein 14 (PP14) was isolated from term human placentae in 1982 by Bohn et al. and has since been shown to inhibit allogeneic MLR (Bolton et al., 1987) and PHA-stimulated lymphocyte proliferation (Pockley et al., 1988). Results from recent research indicate that the immunosuppressive activity of PP14 may be mediated by the suppression of interleukin-1 (IL-1) secretion (Pockley and Bolton, 1990).

A second factor, known as pregnancy zone protein (PZP), is found on the surface of the chorionic villi (Stimson, 1977; Chemnitz et al., 1982). PZP reportedly prolongs the survival of embryonal mouse heart allografts (Svendsen et al., 1978) and suppresses T cell blastogenesis in vitro, possibly by reducing IL-2 production (Saito et al., 1990). However, the significance of PP14 and PZP as immunosuppressors is questionable considering they appear in maternal circulation during

gestation (Julkunen et al., 1985), and systemic immunosuppression would be incompatible with healthy pregnancies.

Ungulate

Conceptus-derived immunosuppressive factors are not exclusively characteristic of decidual type placentae. Factors with similar activities have been reported in several large domestic ungulate species, some of which possess epitheliochorial, cotyledonary placentae. Suppressor molecules have been obtained from uterine secretions of pigs (Murray et al., 1978), sheep (Segerson et al., 1984; Hansen et al., 1987) and cows (Segerson and Libby, 1990) as well as from supernatants of ovine and porcine conceptuses (Murray et al., 1987). While all of the factors share the ability to suppress immune cell function, their immunoregulatory mechanisms and biochemical compositions differ.

Suppression of lymphocytes by bovine (Roberts, 1977) and porcine (Murray et al., 1978) uterine proteins was first reported many years ago. Murray et al. (1978) described the porcine suppressor proteins as small, acidic proteins (approximately 15,000 d) that were present in the uterus at day 15 of the estrous cycle. Subsequent research by other investigators focused more on pregnancy specific proteins and led to the identification of ovine trophoblast protein-1 (Godkin et al., 1984) and bovine trophoblast protein-1 (Godkin et al., 1988). These factors belong to the alpha-interferon family (Imakawa et al., 1987; Stewart et al., 1987), exhibit immunoregulatory activity and are responsible for maternal recognition of pregnancy in their respective species. However, biochemically unrelated factors of a much higher

molecular weight appear to be largely responsible for immunosuppressive activity in uterine/conceptus supernatants.

Segerson et al. (1984) purified two basic glycoproteins (UTM-P) with molecular weights of 57,000 and 59,000 from uterine milk of pregnant ewes (Days 125 and 130 of pregnancy). UTM-P inhibited ^{3}H-thymidine uptake by PHA-stimulated lymphocytes, was not cytotoxic and did not lose its activity with routine handling and storage. Further characterization of uterine milk proteins was carried out using gel filtration (Hansen et al., 1987). The authors reported that the basic protein fraction of uterine secretions contained the suppressive substance and that the substance was large (eluted in the void volume of Sephacryl S-200). Furthermore, the uterine secretions were obtained from non-gravid, ligated uterine horns of pregnant ewes, and thus the factors were identified as being of uterine, and not conceptus, origin.

In the same year, Murray et al. (1987) reported purifying a high-molecular-weight (MW >660,000) acidic glycoprotein (HMWGP) from medium of cultured day 16 and 17 porcine and ovine conceptuses. The HMWGP exhibited a tremendous ability to inhibit ^{3}H-thymidine incorporation by PHA-stimulated lymphocytes, and this activity was not a result of cytotoxicity.

While a number of studies have been carried out to identify and characterize uterine- and conceptus-derived proteins, there is much less information available concerning the suppressive mechanisms of the numerous immunosuppressive factors associated with ungulate pregnancies. Recently, information concerning suppressive mechanisms has been obtained for ovine and bovine uterine luminal proteins (ULP)

(Segerson, 1988; Segerson and Libby, 1990). As these proteins were obtained from uteri of pregnant animals, their specific origin (conceptus or uterine) is unknown. Both ovine and bovine ULP consisted of high (>248,000) and low (14,000-21,000) molecular weight suppressor factors (H-ULP and L-ULP, respectively). Ovine ULP suppressed IL-2 mediated blastogenesis of T lymphocytes and the author suggested that the suppressor mechanism was associated with an alteration of the IL-2 system. Bovine ULP involvement with IL-2 and the IL-2 receptor of T lymphocytes was more extensively investigated (Segerson and Libby, 1990). Using ^{125}I-IL-2 the authors determined that L-ULP did not bind IL-2 but H-ULP bound 70% of ^{125}I-IL-2. Furthermore, a very high-molecular-weight (1.76×10^6 MW) component of H-ULP bound 11.7% of ^{125}I-IL-2 and inhibited binding of ^{125}I-IL-2 to the IL-2R of cow lymphocytes.

Rationale of Present Research

Based on evidence indicating that: 1) the trophoblast plays a critical role in maintaining pregnancy, 2) the maternal immune response is under local suppression at the fetal-maternal interface of successful pregnancies and, 3) immunological rejection is responsible for failed interspecies pregnancies, we hypothesize that factors produced by the trophoblast are important in protecting the fetal allograft and that, during interspecific pregnancy, such factors from trophoblast of one species are frequently not capable of acting effectively on maternal immune cells of another species. To begin testing this hypothesis, the effect of soluble suppressor factors had to be characterized, first on allogeneic lymphocytes and subsequently

on xenogeneic lymphocytes, in order to identify any differences that may be responsible for interspecific pregnancy failure. In the first experiment, the suppressive activity derived from sheep, goat and sheep x goat hybrid trophoblast was examined to deterimine if a decreased suppressive capacity of hybrid tissue could be associated with failure of hybrid pregnancies. Subsequent studies were carried out with horse conceptuses and trophoblast tissue. The results of this research indicate that, similar to that of other species studied, horse trophoblast tissue secretes high-molecular-weight soluble factors capable of inhibiting maternal immune cell function. A >100,000 MW horse trophoblast tissue-derived suppressor factor was identified. The fact that this factor is produced by embryos as early as day 9 and as late as day 26 of gestation, exhibits some degree of species-specificity and acts specifically on T lymphocytes, supports our initial hypothesis. The characterization of this suppressor factor's effect on T cell expression of the IL-2R provides valuable insight into the suppressive mechanism of this factor. These results increase our understanding of the fetal-maternal immunological relationship in the horse and, because these findings are compatible with those reported for other species, it seems likely that the events responsible for fetal allograft protection are largely conserved across species. Therefore, the results of these studies are a significant contribution to the pool of information available to those trying to determine the cause of interspecific pregnancy failure and/or immune-mediated recurrent abortion. In addition, identification of the suppressive mechanism associated with this suppressor factor provides critical information that may be useful in future attempts to

treat recurrent abortion and to facilitate the survival of interspecific pregnancies by altering maternal immune function in utero.

CHAPTER II

SUPPRESSION OF SHEEP AND GOAT LYMPHOCYTE PROLIFERATION BY SHEEP, GOAT AND SHEEP X GOAT HYBRID TROPHOBLAST TISSUE CULTURES[1]

INTRODUCTION

The role of the mammalian placenta in protecting the fetal allograft during pregnancy has not been clearly defined. Although placental tissue expresses antigens early in development (Pavia et al., 1981; Allen et al., 1986; Billington and Burrows, 1986) and the maternal immune system may generate anti-fetal antibodies (Allen et al., 1987; MacLaren et al., 1990), intraspecies pregnancies proceed uninterrupted. It has been suggested that conceptus-derived immunosuppressor factors help protect the fetus from immunological rejection (Mayumi et al., 1985; Clark et al., 1986; Daya and Clark, 1986; Murray et al., 1987; Croy et al., 1988; Roth et al., 1990). The protective mechanism normally involved during intraspecific gestation seems to be ineffective in interspecific and hybrid pregnancies of some closely related species (McGovern, 1975; Frels et al., 1980; Clark et al., 1984). For example, the domestic sheep (*Ovis aries*) x goat (*Capra hircus*) hybrid pregnancy is usually maintained for only 30 to 45 days and there is evidence for immunological involvement in the loss of such pregnancies (Hancock et al.,1968; Dent et al.,1971; McGovern,1973).

This study was designed to determine if medium conditioned with trophoblast tissue of 20 day-old sheep x goat hybrid conceptuses

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[1] Roth, T.L., K.L. White and D.W. Horohov. J. Anim. Sci. (In press).

suppresses sheep and(or) goat lymphocyte proliferation, and to compare hybrid suppressor activity to that of sheep and goat conceptuses.

MATERIALS AND METHODS

Animals. Ovine conceptus donors were Suffolk ewes bred to Merino rams. Does that served as both caprine and hybrid conceptus donors were of various mixed breeds. A Nubian buck and Barbados rams were crossed with does to produce caprine and hybrid conceptuses, respectively.

Superovulation and Breeding Regimen. Estrous cycles of 4 ewes and 8 does were synchronized by inserting intravaginal pessaries containing 60 mg depo-provera (Upjohn, Kalamazoo, MI) for 10 days. To superovulate donors, FSH (Schering Corp., Kenilworth, NJ) was administered every 12 h for 3 days in decreasing doses of 4,4; 3,3 and 2,2 mg, respectively, starting 1 day prior to pessary removal. Donors received an injection of .25 µg Cloprostenol (Estrumate, Bay Vet, Shawnee, KS) coinciding with the second FSH injection.

Animals were observed for estrus twice daily starting on the last day of FSH injections (day 0). Estrous ewes and 4 estrous does were bred by rams and bucks, respectively, every 12 h until no longer in estrus. Similarly, the 4 hybrid donor does were allowed to breed naturally with Barbados rams every 12 h until the end of estrus. To ensure only pregnant donors underwent laparotomies, all mated donor animals were observed for a return to estrus starting 16 days following the last FSH injection. Animals were checked twice daily until the day of conceptus collection (day 20).

Conceptus Collection and Culture. Conceptuses were collected at laparotomy from ewes and does 20 d following their last

FSH injection (day 0). Only those animals that had been in estrus on day 0 were used. Phosphate buffered saline (PBS) supplemented with 1% calf serum (Hyclone, Logan, UT) and 1% antibiotic/antimycotic was used to flush pregnant uteri. PBS was injected into the uterine body at the base of each horn and conceptus tissue was collected via retrograde flushing through a glass cannula (12 mm i.d.) inserted into the tip of the uterine horn. Conceptuses were collected from nine animals, three donors of each conceptus type (caprine, ovine and hybrid). Conceptuses were immediately rinsed three times in sterile PBS and all fetuses were removed from tissue. Tissue was rinsed once in RPMI-1640 (Hyclone, Logan, UT) supplemented with 1% antibiotic/antimycotic and 15% fetal calf serum (Hyclone, Logan, UT; non-heat-treated, <0.1 ng/ml endotoxins) (RPMI+) and was divided into two or three approximately equal sections. Each tissue section was placed in a tissue culture flask (25 cm^2) containing 15 ml RPMI+. Conceptus tissue cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 and air for 24 h. To obtain trophoblast tissue-conditioned medium, the content of each culture was poured into a 15 ml conical centrifuge tube and centrifuged (600 x *g*; 30 min). Supernatant was filtered through a .22 µm filter and was stored in 1 ml aliquots at -80°C. The tissue from each culture was blotted dry and weighed. Average weights for sheep (n=3), goat (n=3) and hybrid (n=3) tissues were .34, .33 and .34 gm/flask, respectively.

Lymphocyte Proliferation Assay. Whole blood was collected into heparinized tubes by jugular venipuncture from 4 randomly chosen ewes and does. The blood was diluted with an equal volume of Hank's basic salt solution (HBSS; Hyclone, Logan, UT) and 7 ml of the

blood/HBSS mixture was layered over 4 ml Ficoll-Paque (Pharmacia, Piscataway, NJ). Following centrifugation in a 15 ml centrifuge tube (550 x *g*; 30 min), the interface cells were collected and washed twice with HBSS. The cell pellet was resuspended in RPMI+ medium, cells were counted on a hemacytometer and cell suspensions were diluted with RPMI+ to 4 x 10^6 cells/ml.

Lymphocyte cultures were carried out in triplicate in 96-well round-bottomed tissue culture-treated plates (Corning Glass Works, Corning, NY). All wells received 1 x 10^5 cells (25 µl). Non-stimulated and stimulated controls received 75 and 50 µl RPMI+, respectively, and stimulated controls also received 25 µl RPMI+ containing .1 µg PWM. Experimental wells were supplemented with 6, 3, 1.5 or .75 µl of caprine, ovine or hybrid conditioned medium (CCM, OCM and HCM, respectively) diluted in 50 µl RPMI+. For each type of conditioned medium, samples of three cultures obtained from three different donors were tested. All experimental wells also received 25 µl aliquots of RPMI+ containing .1 µg PWM. Total volume in all wells was 100 µl. All cultures were carried out simultaneously using the same cell suspensions and cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 and air. ^{3}H-thymidine (1 µCi/well; 6.7 Ci/mmol, ICN, Irvine, CA) was added to cultures 96 h after the start of incubation, and cells were harvested at 112 h with a Skatron automatic cell harvester (Flow Laboratories, Rockville, MD). Radioactivity, a measure of cell proliferation, was determined by liquid scintillation counting. Prior to harvest, percent cell viability was determined in wells of each treatment group by trypan blue exclusion.

Statistical Analysis. Data reported as percent of stimulated control was calculated for triplicate wells using the equation (% control = average cpm of experimental cultures / average cpm of stimulated control cultures X 100). Results were analyzed in a 3 x 4 factorial ANOVA (3 conceptus types and 4 volumes of conditioned medium) using the general linear model procedures of the statistical analysis systems (SAS) computer package (Luginbuhl et al., 1985). Random variables were lymphocytes and conceptuses. Highly insignificant ($P>.75$) interactions were deleted from the model and appropriate error terms were used to test the main effects, i.e. variation attributed to species of lymphocyte was tested with the error term lymphocyte within species and variation due to type of conceptus was tested using conceptus within type. Tukey's mean comparison tests were used to compare the effects of different volumes of conditioned medium and to determine differences between sheep and goat lymphocyte suppression within CCM, HCM and OCM treatment groups.

RESULTS

The superovulation and breeding regimen proved effective in that, with the exception of one goat conceptus donor, all donor animals exhibited numerous corpora lutea and multiple conceptuses were obtained from each animal. When compared to goat and sheep conceptuses, hybrid conceptus tissue appeared healthy and fetal development normal, indicating that failure of hybrid pregnancy had not yet occurred.

Medium conditioned with ovine, caprine and hybrid trophoblast tissue suppressed ($P<.01$) both sheep and goat PWM-stimulated lymphocyte proliferation in a dose-dependent manner (Figures 1 and 2).

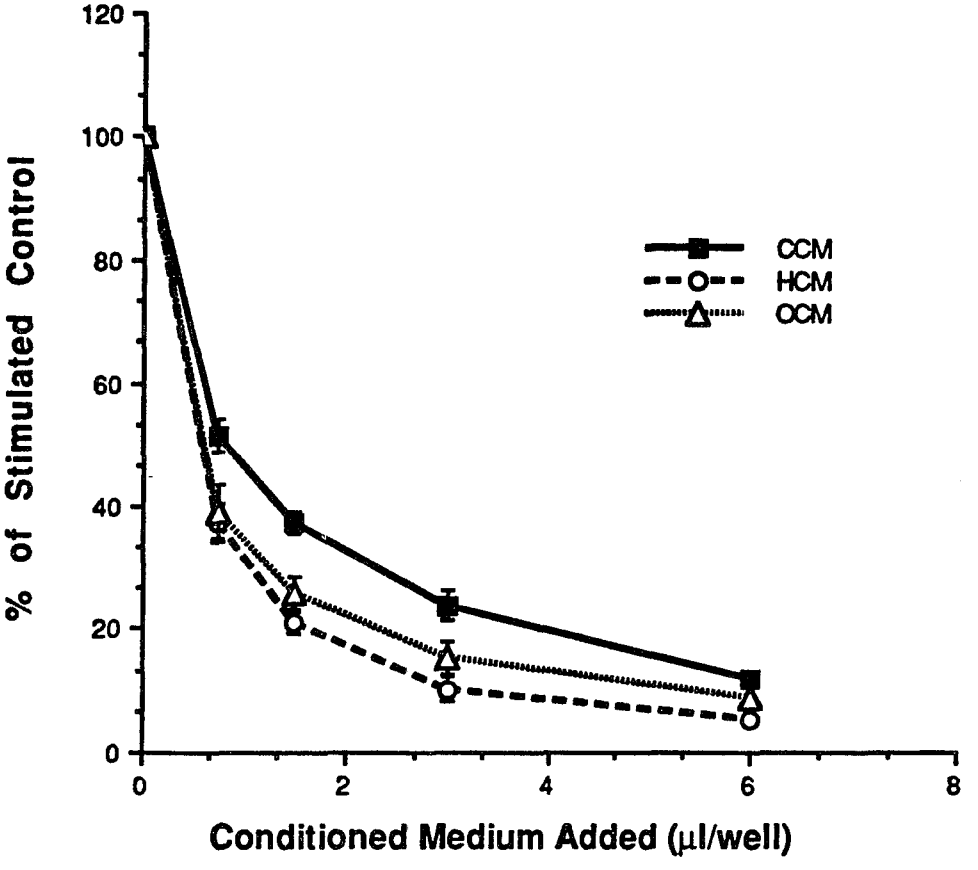

Figure 1. The effect of caprine, ovine and hybrid trophoblast tissue-conditioned medium (CCM, OCM and HCM, respectively) on PWM-stimulated sheep lymphocyte proliferation. Values representmean percent of stimulated control ± SEM (n=12). Overall suppressor activity did not differ (P>.05) between treatments.

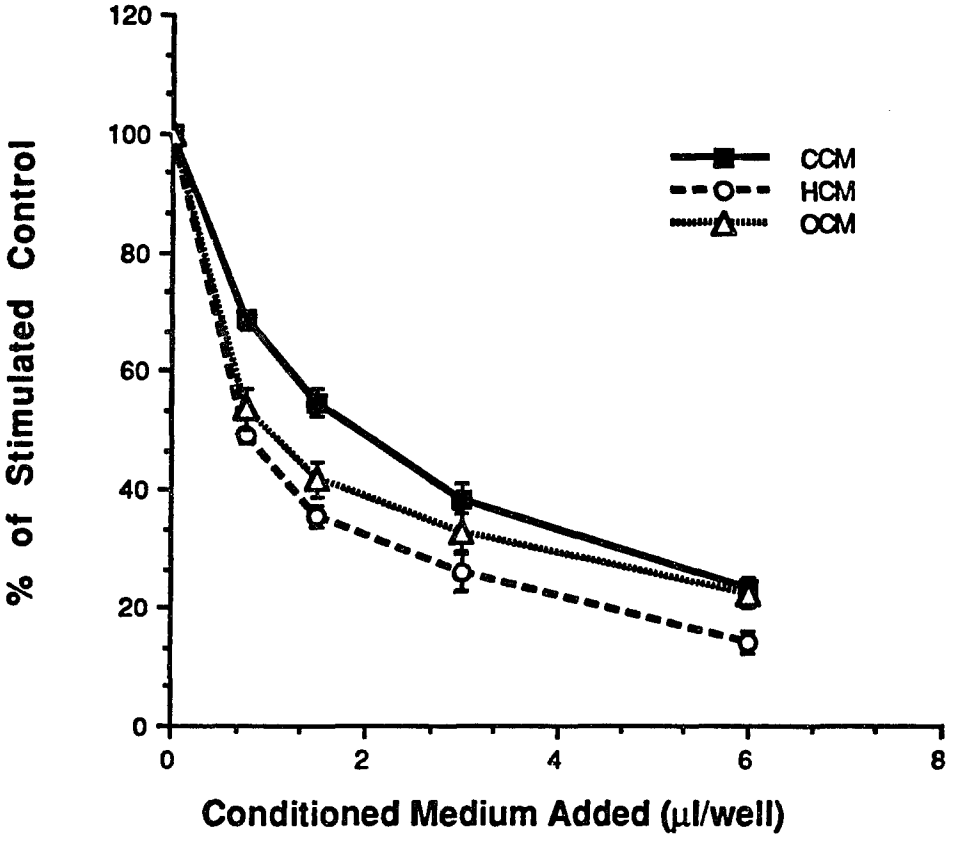

Figure 2. The effect of caprine, ovine and hybrid trophoblast tissue-conditioned medium (CCM, OCM and HCM, respectively) on PWM-stimulated goat lymphocyte proliferation. Values represent mean percent of stimulated control ± SEM (n=12). Overall suppressor activity did not differ (P>.05) between treatments.

The overall suppressor activity of CCM, OCM and HCM on lymphocytes of either sheep or goats did not differ (P>.05). Data comparing sheep and goat lymphocyte proliferation within each type of conditioned medium are in Table 1. The results indicate that proliferation of sheep lymphocytes was suppressed more than that of goat lymphocytes when treated with CCM, OCM or HCM (P<.01, P<.01 and P<.05, respectively). Viability of lymphocytes at the end of culture was consistently 85-95% across all treatments.

DISCUSSION

Evidence supporting the theory that the placenta plays an important role in maintaining the fetus as an allograft in the mother has been obtained through the use of embryo manipulation techniques. The injection of embryonic cells from one species into blastocysts of a different species can result in successful interspecies pregnancies provided the placenta and recipient are of the same species (Rossant et al., 1982; Fehilly et al., 1984; Meinecke-Tillman and Meinecke, 1984; Polzin et al., 1987). This technique has been used to produce healthy offspring containing sheep x goat hybrid cells (Roth et al., 1989). These results indicate that the hybrid placental tissue, not the fetal cells, is responsible for the failure of sheep x goat hybrid pregnancies. One explanation for this failure may be a deficiency in the ability of hybrid trophoblast tissue to create and maintain an immunosuppressive environment at the fetal-maternal interface.

While there have been a number of reports concerning immunosuppressive activity in ovine uterine fluid during intraspecies pregnancies (Hansen et al., 1987; Segerson et al., 1984; Segerson, 1988), there has been less published data pertaining to ovine

TABLE 1. Suppression of PWM-stimulated sheep and goat lymphocyte proliferation by caprine, hybrid and ovine trophoblast tissue-conditioned medium (CCM, HCM and OCM, respectively)[a].

	Conditioned medium[b]					
	CCM[c]		HCM[c]		OCM[c]	
Medium (µl/well)	Sheep	Goat[d]	Sheep	Goat[e]	Sheep	Goat[d]
.75	51.6 ± 2.6	68.8 ± 1.5	37.3 ± 3.2	49.2 ± 1.2	39.1 ± 4.3	53.7 ± 3.3
1.5	37.3 ± 1.9	54.7 ± 2.3	21.2 ± 1.7	35.6 ± 1.8	25.8 ± 2.7	41.8 ± 2.8
3	23.8 ± 2.4	38.5 ± 2.5	10.3 ± 1.8	26.1 ± 3.1	15.5 ± 2.7	32.8 ± 3.1
6	12.0 ± 1.1	23.3 ± 2.0	5.3 ± 1.0	14.1 ± 2.0	9.0 ± 1.7	22.3 ± 2.4

[a]Values represent (mean ± SEM) percent of sheep (n=4) and goat (n=4) stimulated controls (109,000 and 183,000 cpm, respectively). Non-stimulated control (background) values for sheep and goat were 5,330 and 6,100 cpm, respectively.

[b]Medium conditioned by the 24 h culture of 20 d-old caprine, hybrid and ovine trophoblast tissue (CCM, HCM and OCM, respectively).

[c]Tukey's mean comparison test was used to determine differences in overall sheep and goat lymphocyte proliferation within the conditioned medium.

[d]Suppression of goat lymphocyte proliferation was less ($P<.01$) than that of sheep lymphocytes.

[e]Suppression of goat lymphocyte proliferation was less ($P<.05$) than that of sheep lymphocytes.

conceptus-derived suppression (Murray et al., 1987; Staples et al., 1983) and information is lacking regarding that of the sheep x goat hybrid conceptus. In this study, we focused on the production of immunosuppressor factors by the developing placenta. The cultures contained conceptus trophoblast tissue devoid of the amnion, which was discarded with the fetus. Therefore, the immunosuppressive activity of CCM, OCM and HCM, reported herein, was derived specifically from early placental tissue of 20 day-old goat, sheep and sheep x goat hybrid conceptuses, respectively.

Conceptuses were collected on day 20 of gestation for two primary reasons. First, because hybrid pregnancy loss occurs between days 35 and 45 of gestation (Hancock et al., 1968; Tucker et al., 1971; McGovern, 1973) and has been reported as early as day 30 (Hancock et al., 1968), we wanted to ensure that healthy conceptus tissue was obtained. Our collection of numerous, apparently normal hybrid conceptuses was not unexpected as others have reported normal hybrid fetal and placental development until day 30 to 35 of pregnancy (Hancock and Jacobs, 1966; Hancock et al., 1968), beyond which excessive lymphocyte infiltration (Hancock et al., 1968) and increased allantoic fluid accumulation occurs (McGovern, 1977). The second reason for collecting day 20 conceptuses was based on the hypothesis that these reported abnormal characteristics result from a disruption of the immunoregulated uterine environment. Such a disruption would likely occur some time prior to the manifestation of these abnormalities. Therefore, if conceptus-derived suppressor factors are involved with controlling uterine immune function, there may be some

change in the immunosuppressive competence of the hybrid conceptus as early as day 20 of gestation.

The results of this study indicate that HCM effectively suppressed in vitro sheep and goat lymphocyte proliferation and, therefore, the findings fail to support the hypothesis that a lack of conceptus-derived immunosuppressive activity precedes rejection of the hybrid conceptus. However, a decrease in immunosuppressive activity may occur closer to the time of fetal death and simply cannot be detected on day 20 of gestation. Alternatively, the production and(or) functions of conceptus-derived suppressor factors may be altered while in vivo via fetal-maternal tissue interactions, but once removed from the uterine environment and placed in culture, the production and(or) functions of these factors resume. Finally, it is possible that suppressor factors are consistently produced during early hybrid gestation, but other events lead to maternal lymphocyte escape from suppression and, perhaps, to their attack of the hybrid conceptus.

Maternal lymphocyte evasion of suppression may be mediated via strong antigenic stimulation. Xenogeneic antigens expressed on hybrid and interspecific placental tissue may have more stimulatory impact on the maternal immune system than allogeneic fetal antigens, thereby disrupting the seemingly compatible fetal-maternal relationship that normally exists during intraspecific pregnancy. Although much is still unknown about this intimate relationship between maternal and fetal tissue, in the two most intensively studied models of interspecies and hybrid pregnancy (murine and equine) the immune response seems to play an integral role in implantation and placentation. Murine embryo implantation is associated with lymphocyte accumulation at the fetal-

maternal interface (Slapsys and Clark, 1982; Slapsys and Clark, 1983) but only in unsuccessful interspecies or hybrid pregnancies does the trophoblast become infiltrated with lymphocytes (Croy et al., 1982). In intraspecific equine pregnancies, lymphocytes accumulate around the endometrial cups but do not penetrate into the cup tissue until days 70 to 80 of gestation (Allen, 1982). However, in equine hybrid pregnancies, lymphocyte invasion of the cups occurs much more rapidly with complete loss of endometrial cups occurring at day 60 (Allen, 1969; Allen, 1982). Similarly, the excessive lymphocyte infiltration observed in sheep x goat hybrid pregnancies (Hancock et al., 1968) is in contrast to a noted decrease in accumulated uterine lymphocytes following attachment of the conceptus in intraspecific ovine pregnancies (King et al., 1982). Together, these data may indicate a local suppression of the maternal immune cells at the fetal-maternal interface is ineffective in hybrid and interspecific pregnancies probably due to a disruption of suppressor activity and(or) an overriding immune response against xenogeneic antigens. At day 20 of pregnancy, the ovine and caprine conceptus has attached (Leiser, 1975; King et al., 1982) but interdigitation of chorionic microvilli in developing placentomes does not occur until the fourth week of gestation (Boshier, 1969; Dent, 1973). As this event is likely to provide the antigenic stimulation that may initiate changes leading to maternal immunological rejection, it is possible we obtained conceptuses prior to any immunological alterations in the uterine environment that could affect subsequent placental suppressor activity.

HCM suppressed sheep and goat lymphocytes as effectively as OCM and CCM. However, it is important to point out that PWM was used to stimulate lymphocyte proliferation and, in a previous study involving horse conceptus-derived suppression, Roth et al.(unpublished data) found that PWM-stimulated lymphocytes were easier to suppress than phytohemagglutinin- or lipopolysaccharide-stimulated lymphocytes. These results indicate that the type of antigen stimulating the lymphocytes plays a role in determining the extent to which proliferation is suppressed by conceptus suppressor factors. In utero, lymphocytes stimulated by hybrid placental xenoantigens may be more difficult to suppress than those stimulated with intraspecific fetal alloantigens. Therefore, the apparent equality of suppressive activity of HCM, OCM and CCM in in vitro PWM-stimulated lymphocyte assays may not be an accurate assessment of the in vivo situation. Unfortunately, an in vivo analysis of lymphocyte-conceptus tissue interaction was beyond the scope of this study.

The reported difference between sheep and goat lymphocyte suppression within each treatment (Table 1) is likely a reflection of the difference in species responsiveness to the PWM. To ensure consistency across all assays, the same PWM concentration (.1 µg/well) was supplemented to all stimulated cultures. This concentration is one that provokes a strong proliferative response in lymphocytes of both species. However, when a mitogen titer assay was carried out for goat and sheep lymphocytes, the optimal lymphocyte responses were achieved using .1 and .2 µg/well, respectively (unpublished data). Thus, the PWM dose used in the assay was slightly suboptimal for sheep lymphocytes, which may explain the lower stimulated control values and

the greater suppression of sheep lymphocytes with CCM, OCM and HCM when compared to that of goats.

In summary, superovulation of does followed by natural mating with Barbados rams may result in numerous hybrid conceptuses with apparently normal fetal development and healthy trophoblast tissue at day 20 of gestation. Medium conditioned with hybrid trophoblast tissue will suppress proliferation of PWM-stimulated sheep and goat lymphocytes. No differences between suppressive activity of CCM and OCM or HCM obtained from 20 day-old conceptuses was determined. These results indicate that, at day 20 of gestation, sheep x goat hybrid trophoblast does not differ from ovine or caprine trophoblast in its ability to suppress PWM-stimulated lymphocytes.

CHAPTER III

SUPPRESSION OF LYMPHOCYTE PROLIFERATION BY A >30,000 MW FACTOR IN HORSE CONCEPTUS-CONDITIONED MEDIUM[2]

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

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[2] Roth, T.L., K.L. White, D.L. Thompson, Jr., B.E. Barry, J.S. Capehart, D.R. Colborn and M.H. Rabb. 1990. Biol. Reprod. 43:298-304.

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; McLaren 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 immunological 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 pre-implantation 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 x 10^6 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 non-surgical embryo collection procedure previously described by Imel et al. (1981). Ultrasonography was used on day 12 to determine pregnancy in Group II and III conceptus donor mares. Three Group II and III conceptuses from six different mares were recovered by a modified non-surgical uterine flushing technique. Mares were lightly sedated with 1-3 ml Rompun (Mobay, Shawnee, KS) administered iv five minutes prior to initiating the flushing procedure. The mare's cervix was mechanically dilated 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+ and were placed in tissue culture flasks containing RPMI+ as follows: Group I - two embryos/4 ml, Group II - one conceptus/12 ml and Group III - one conceptus/30 ml. All cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2 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 .2 µm filter.

Lymphocyte proliferation assay. Heparinized whole blood, obtained by jugular venipuncture from 3 randomly chosen mares served as a source of peripheral blood lymphocytes (PBLs). Blood from each animal was separately mixed with an equal volume of HBSS. Ten ml of the blood-HBSS mixture were layered over 4 ml Ficoll-Paque in a 15 ml conical tube and samples were centrifuged for 20 min at 500 x g. Interface cells from each horse were pipetted into separate centrifuge tubes and rinsed twice with 8 ml HBSS (400 x g; 10 min). The peripheral blood mononuclear cells were resuspended in RPMI+, counted on a hemacytometer and diluted to a final concentration of 4 x 10^6 cells/ml. Cultures were carried out in triplicate in 96-well round-bottom tissue culture treated plates. All wells received 1 x 10^5 cells (25 µl). Stimulated cultures received 25 µl aliquots of RPMI+ containing one of three mitogens: 1 µg PHA, 1 µg PWM or 5 µg Con A. HCCM from Groups I and II was 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. Non-stimulated 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 with 5% CO_2 and air. ^{3}H-thymidine was added to cultures (1 µCi/well) at 96 ± 2 h and cells were harvested 16 ± 2 h later onto glass fiber filters using a Skatron automatic cell harvester. 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 inter- assay variability and to increase assay sensitivity, mitogen titer assays were run on lymphocyte donors to determine the optimal mitogen dose for each donor. PWM was serially diluted from 4 to .05 µg/well and three mares responding optimally to the same mitogen dose of .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 .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 .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 centrifuged at 4500 RPM for 30 min. through amicon microconcentrators (Amicon, Danvers, MA) with MW exclusions of 10,000 and 30,000. Sample concentrates were rinsed by diluting with 500 µl RPMI-1640 and spinning again at 4500 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 effect 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 .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 .4, .8, 1.6 , 3.2 , 6.4 and 12.8 µl/well (total well volume = 100 µl).

Statistical Analysis. Average cpm and % of stimulated control for triplicate or quadruplicate wells were determined. Results (cpm) from the addition of HCCM of Group I, II and III cultures to lymphocyte proliferation assays were analyzed by ANOVA using the general linear model procedures of the 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 interspecies 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 ($P<.001$) induced by PWM, while CM exhibited no suppressive activity ($P>.05$) when similarly added to cultures (Table 2). In all groups, there was a linear relationship ($P<.01$) between % suppression and the addition of 0, 25 and 50% HCCM. HCCM also suppressed lymphocyte stimulation ($P<.001$) induced by Con A and PHA 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.

TABLE 2. Effect of control medium and HCCM from Groups I-III on mitogen-stimulated horse PBL proliferation.[a]

Source of Medium	(%Vol)	Mitogen Stimulation (cpm) None	PWM[b]	ConA[c]	PHA[b]
Group I	0	6,896 ± 2,299			
(Day 9-10)	0		37,504 ± 6,240		39,260 ± 4,423
	25		21,745 ± 3,986*		19,882 ± 3,198*
	50		19,312 ± 3,668*		17,816 ± 2,843*
Group II	0	35,286 ± 5,377			
(Day 15-16)	0		85,480 ± 5,724	53,783 ± 4,431	
	25		22,547 ± 5,067*	23,647 ± 3,971*	
	50		9,766 ± 2,428*	11,349 ± 2,262*	
Group III	0	14,018 ± 2,194			
(Day 25-26)	0		61,899 ± 5,384		
	25		33,193 ± 6,179*		
	50		19,975 ± 4,706*		
Control Medium	0	6,896 ± 2,299			
	0		37,504 ± 6,240		
	25		43,462 ± 6,230		
	50		43,197 ± 5,823		

[a]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). Group 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 prolferation ($P<.001$) in mitogen-stimulated cultures, but CM did not ($P>.05$).

[b]Supplemented to cultures at 1 µg/well.

[c]Supplemented to cultures at 5 µg/well.

*Significant lymphocyte suppression relative to stimulated controls ($P<.001$).

HCCM Characterization. After treatment (HT, FT, NCF), the addition of increasing volumes of HCCM suppressed ($P<.05$) PWM-induced lymphocyte proliferation in a dose-dependent manner (Table 3). Similarly treated CM did not suppress ($P>.05$) proliferation (data not shown). There was no difference in overall suppressive activity (determined as % of stimulated control) of HCCM between treatments, nor did suppressive activity of treated samples differ ($P>.05$) from that of non-treated (FF) HCCM (Fig. 3).

The fractions of HCCM separated by 10,000 and 30,000 MW exclusion amicon microconcentrators suppressed ($P<.01$) lymphocyte proliferation with the larger MW HCCM fraction significantly more suppressive ($P<.01$) than the smaller MW fraction (Figs. 4 and 5). 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 centricon), and 77.2 ± 5.1 and 28.4 ± 9.1 respectively (30,000 MW exclusion centricon), while 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 centricon) and 112.3 ± 1.0 and 108.5 ± 7.3 (30,000 MW centricon)).

Interspecies Assay. The addition of HCCM to goat, donkey and horse PWM-stimulated lymphocytes decreased ($P<.05$) lymphocyte proliferation across all species (Fig. 6). Overall suppression (determined relative to stimulated controls) was less in goat lymphocyte cultures than that in horse or donkey cultures ($P<.05$), 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

TABLE 3. Effect of treated HCCM on PWM-stimulated horse PBL proliferation.[a]

HCCM added (µl/well)	HCCM Treatment: Fresh-Frozen	Frozen-Thawed	Heat-Treated	Nitrocellulose Filtered
0.0	70,483 ± 3,844	73,645 ± 5,156	77,985 ± 4,719	39,048 ± 9,774
0.75	36,370 ± 473	30,281 ± 684	33,363 ± 1,439	21,662 ± 5,735
1.56	20,959 ± 1,504	21,098 ± 1,587	21,790 ± 1,910	14,645 ± 4,427
3.125	15,075 ± 1,076	13,400 ± 1,286	9,627 ± 1,817	8,078 ± 2,787
6.25	8,986 ± 881	11,707 ± 1,007	5,382 ± 1,641	4,424 ± 1,914
12.5	5,107 ± 457	5,178 ± 556	2,904 ± 362	2,463 ± 1,097
25.0	3,056 ± 248	3,184 ± 283	1,527 ± 122	1,434 ± 580
Non-stimulated	14,579 ± 2,208	12,843 ± 3,122	13,998 ± 3,402	2,951 ± 1,316

[a]Data represent mean cpm ± SEM (n=3). All samples expressed suppressive activity ($P<.05$) relative to stimulated controls (0.0 µl/well).

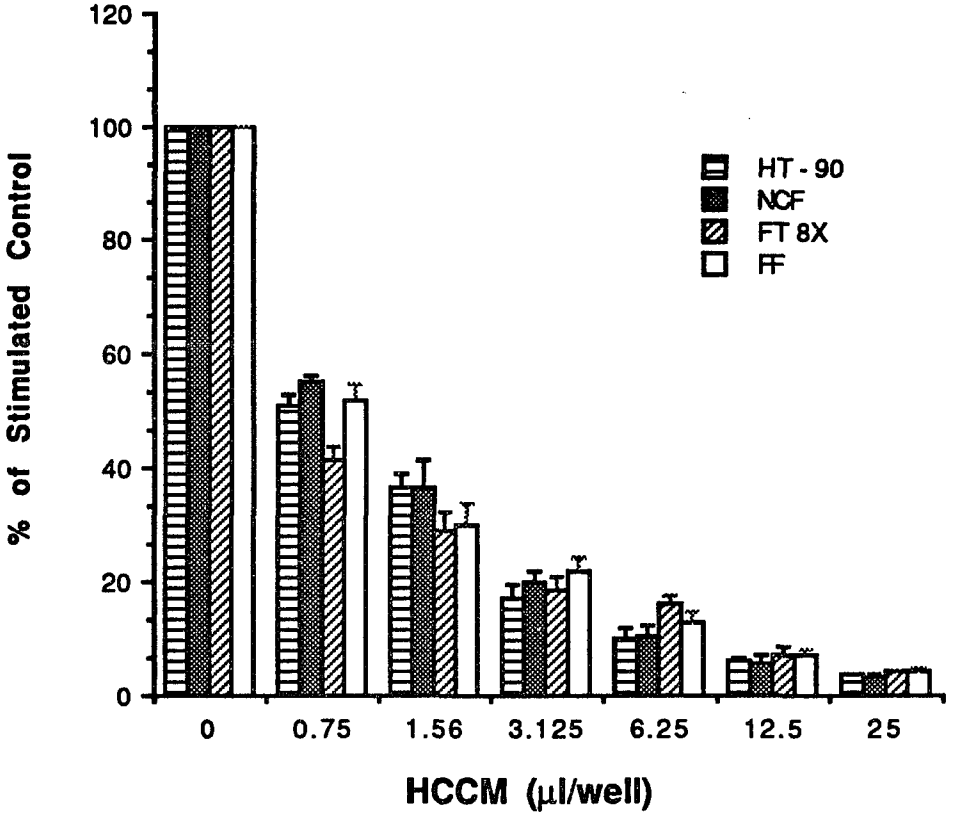

Figure 3. Suppressive activity of treated (heat-treated 90°C for 30 min (HT), filtered 2x through a .2 µm nitrocellulose filter (NCF), frozen to 80°C and thawed to room temperature 8x (FT)) vs non-treated fresh-frozen (FF) HCCM on PWM-stimulated PBL proliferation. Columns represent mean % of control + SEM (n=3) with the addition of increasing quantities of HCCM. All samples expressed suppressive activity ($P<.05$), and there was no difference ($P>.05$) between the activity of treated and non-treated HCCM.

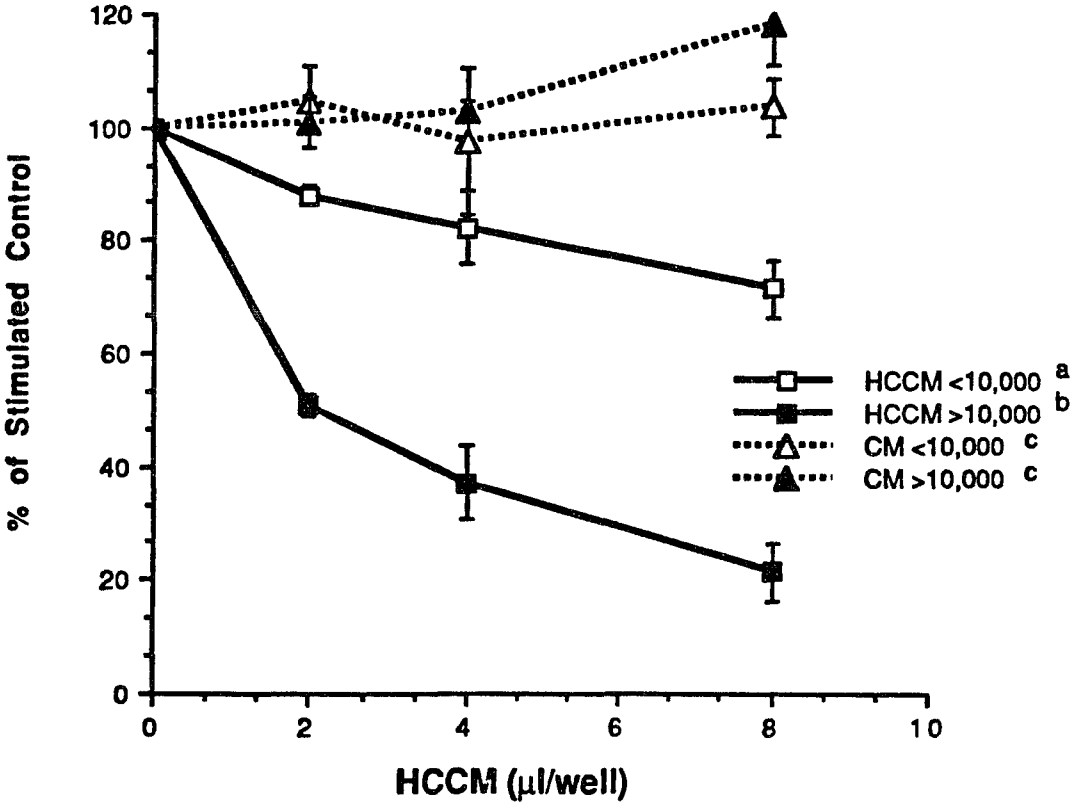

Figure 4. Effect of >10,000 MW and <10,000 MW fractions of HCCM and CM on PWM-stimulated PBL. Fractions were separated by amicon microconcentrator centrifugation. Values represent mean % of stimulated control ± SEM (n=3). Both HCCM fractions were suppressive compared to CM fractions ($P<.05$). Different superscripts denote significantly different suppressive activity ($P<.05$).

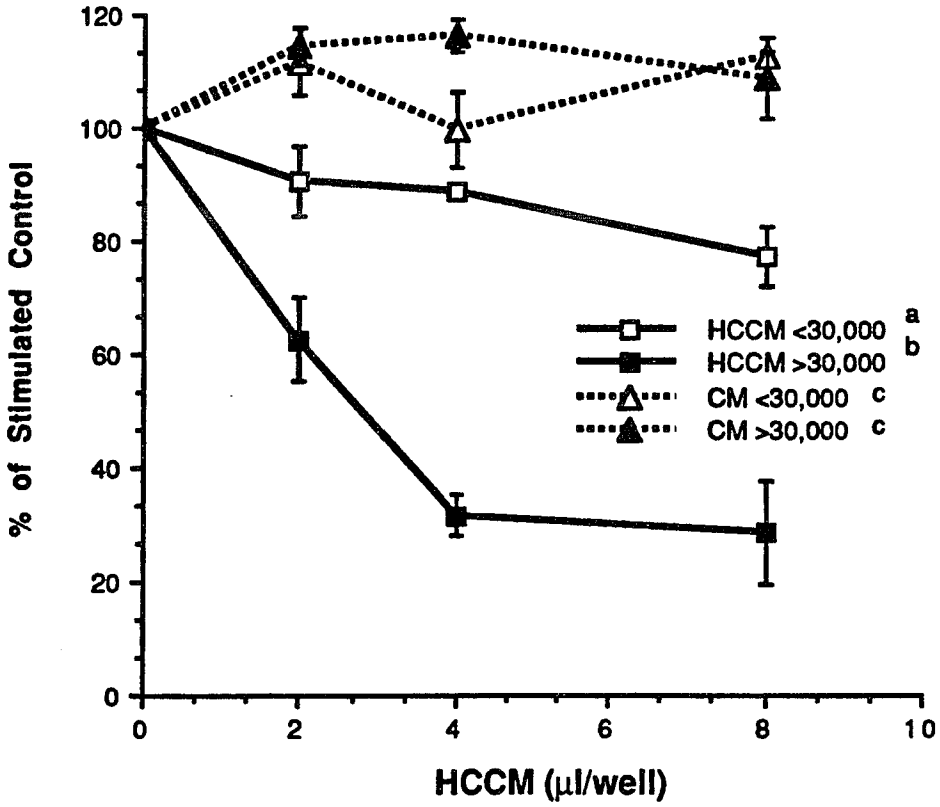

Figure 5. Effect of >30,000 MW and <30,000 MW fractions of HCCM and CM on PWM-stimulated PBL. Fractions were separated by amicon microconcentrator centrifugation. Values represent mean % of stimulated control ± SEM (n=3). Both HCCM fractions were significantly suppressive ($P<.05$) compared to CM fractions. Different superscripts denote significantly different suppressive activity ($P<.05$).

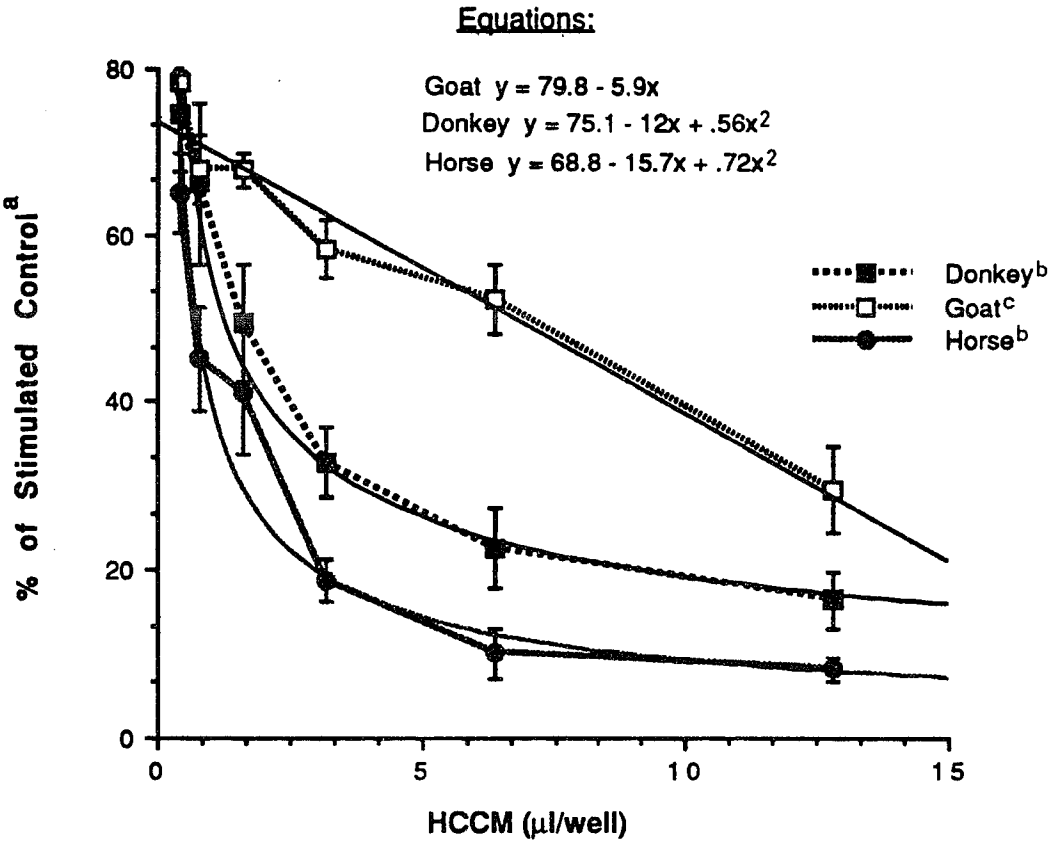

Figure 6. Effect of HCCM on PWM-stimulated PBL of horses, donkeys and goats. Values are mean % of stimulated control ± SEM (n=3) of PBL with the addition of increasing quantities of HCCM. Overall suppression of goat PBL was less ($P<.05$) than that of horse or donkey PBL. There was no difference ($P>.05$) 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; $R^2=.88$).

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

[b,c]Different superscripts denote significant differences in overall suppression caused by HCCM supplementation ($P<.05$).

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 while the goat's best fit a linear equation.

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 pellucidae 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 non-detectable prior to day 10, variable from day 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 levels 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 greatest 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 recipients. However, in the cow such an assay would not be practical since suppressor activity of embryos is not detected until after day 10, and embryo transfer in cattle is carried out between days 6 and 8. The transfer of embryos at day 9 in the horse is successful, and since equine embryos 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. Group II and III is likely simply due to the difference in the tissue:medium ratio in the cultures, resulting in a lower concentration 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 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 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 that lymphocyte proliferation was not affected by the addition of CM to the lymphocyte cultures ensures 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 in order to achieve suppression of PHA and Con A stimulated lymphocyte proliferation (Mori et al., 1977; Siiteri and Stites, 1982). Only picogram to nanogram concentrations of hormones are found in equine blastocoelic fluid or in equine conceptus culture medium (Flood et al., 1979; Marsan 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 smaller MW fractions.

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 stabile structure perhaps due to many disulfide bridges. It is also possible the substance does undergo structural denaturation but 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 horse 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 oTP-1 and bTP-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 anti-viral activity has also been identified, and it too is small

(22,000-24,000 M_r) 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 exhibited greater suppressive activity when added to lymphocytes of the same species as 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 and this may explain, in part, how donkeys are able to carry horse fetuses to term. When added to goat lymphocytes, however, HCCM is much less suppressive indicating that with more distantly related species the HCCM suppressor factor is not as efficient at inhibiting

lymphocyte proliferative responses. Furthermore, both donkey and horse lymphocyte suppressor curves followed quadratic functions with the same slope indicating that the mechanism of action of the suppressor substance in HCCM is similar on lymphocytes of the two species. With the much less-related caprine species, the HCCM suppressor substance appears to be acting in a different manner resulting in a suppressor curve that follows a linear function. Although it is suppressive, HCCM is much less suppressive when added to lymphocytes of the caprine species, and also appears to be suppressing the lymphocytes through a much less effective mechanism. Although these data provide evidence for species-specific activity of HCCM, we cannot ignore the possibility that goat lymphocytes may be inherently more difficult to suppress than equine lymphocytes when stimulated with PWM.

In summary, we have found that from day 9 through day 26 of development the equine conceptus produces an immunosuppressive factor that inhibits *in vitro* horse lymphocyte proliferation in a non-antigen specific manner. The suppressor factor appears to be >30,000 MW and maintains full biological activity after repeated freeze-thaw and after heating to 90^{o}C for 30 minutes. This substance exhibits its suppressive effect in a similar manner on donkey and horse lymphocytes, yet on lymphocytes of the less-related goat species, suppressive activity is significantly decreased. These data indicate that the substance is, to some degree, species-specific. Determining the significance of this immunoregulatory substance in maintaining the conceptus, and its possible relationship to successful interspecies pregnancies should help in our understanding of the puzzling phenomenon of fetal allograft survival.

CHAPTER IV
PGE_2-INDEPENDENT IMMUNOSUPPRESSIVE ACTIVITY OF HORSE TROPHOBLAST TISSUE[3]

INTRODUCTION

Suppression of the maternal immune response at the fetal-maternal interface may be imperative for the survival of the allogeneic conceptus during pregnancy. Immunosuppressive factors associated with placental and/or uterine tissues and their secretions have been reported for several species, including mice (Clark et al., 1985,1986; Chaouat, 1987), humans (Daya and Clark, 1986; Pockley et al., 1988), sheep (Segerson et al., 1984; Hansen et al., 1987; Murray et al., 1987; Roth et al., 1991), goats (Roth et al., 1991), pigs (Murray et al., 1978), cows (Roberts, 1977; Segerson and Gunsett, 1990; Segerson and Libby, 1990) and horses (Roth et al., 1990). In addition, immune cells with suppressor activity accumulate in and around the placenta (Slapsys and Clark, 1982,1983; Clark et al., 1983; Brierley and Clark, 1985; Uren and Boyle, 1990). Both placental suppressor cells and decidual cells, produce the immunosuppressive prostaglandin, PGE_2 (Scodras et al., 1990). Likewise, early developing embryos of several species produce PGE_2 (Lacroix and Kann, 1982; Pakrasi and Dey, 1982; Hwang et al., 1988; Holmes et al., 1990). PGE_2 suppresses IL-2 production by T lymphocytes (Rappaport and Dodge, 1982; Walker et al., 1983) and induces T suppressor cells (Chouaib and Fradelizi, 1982; Delfraissy et al., 1982; Kaszubowski and Goodwin, 1982). Therefore, it has been suggested that PGE_2 is an important immunosuppressant of pregnancy. The fact that PGE_2 from decidual cells

[3] Roth, T.L., K.L. White, D.L. Thompson, Jr. and D.W. Horohov. J. Reprod. Immunol. (In review).

and decidual macrophages inactivates T lymphocytes (Parhar et al., 1989) and natural killer (NK) cells in the decidua, (Scodras et al., 1990) supports the theory that PGE_2 activity is conducive to fetal survival (Lala et al., 1990).

We reported that horse conceptus-conditioned medium suppresses in vitro lymphocyte proliferation in vitro (Roth et al., 1990). The specific objective of this study was to determine if indomethacin, a prostaglandin inhibitor, could reduce horse trophoblast-derived immunosuppressive activity.

MATERIALS AND METHODS

Animals and conceptus collections. Conceptuses were obtained from mares on day 21 of gestation according to procedures described in Chapter III. The flushing medium consisted of sterile PBS supplemented with 1% penicillin/streptomycin (Gibco, Grand Island, NY).

Conceptus cultures. Three conceptuses, each from a different mare, were obtained on day 21 of gestation. Under sterile conditions, conceptuses were rinsed twice with PBS. Both the embryonic capsule and the fetus proper were removed from the trophoblast tissue of each conceptus. The trophoblast tissue was cut into two approximately equal sections and each section was rinsed twice in sterile RPMI-1640 medium supplemented with 1% antibiotic/antimycotic (RPMI). Each trophoblast section from each conceptus was randomly assigned to 2 treatments. For treatment 1, each tissue section was placed in a tissue culture flask (25 cm^2) containing 5 ml RPMI. Treatment 2 tissue sections were each placed in a flask with 5 ml RPMI containing 1×10^{-4} M indomethacin (Sigma, St. Louis, MO). An additional flask

was set up with 5 ml indomethacin control medium (I-CM) which consisted of RPMI with 1 x 10^{-4} M indomethacin but no trophoblast tissue. A total of 6 trophoblast tissue cultures (3 in RPMI and 3 in RPMI + indomethacin) and a seventh flask containing I-CM were incubated for 28 h at 37°C in a humidified atmosphere of 5% CO_2 and air. Following incubation, horse trophoblast-conditioned medium (HTCM) and indomethacin-treated trophoblast-conditioned medium (I-HTCM) were obtained by first pouring the contents of each flask into a conical tube and then centrifuging the tubes at 1,500 x *g* for 10 min. Supernatants were filtered through a .2 µm filter and were stored in 1 ml aliquots at -80°C. Following centrifugation, the tissue volume in each culture was determined. The volume was consistent, measuring approximately 100 µl of tissue in each culture. I-CM was similarly aliquoted and stored at -80°C.

Lymphocyte proliferation assay. Peripheral blood lymphocytes were obtained as described in chapter III. Cultures were carried out in quadruplicate in 96-well, round-bottomed tissue culture-treated plates. Non-stimulated control cultures contained 25 µl cell suspension (1 x 10^5 cells) and 75 µl RPMI+. Stimulated control wells received 25 µl cells, 25 µl RPMI+ containing 0.1 µg pokeweed mitogen (PWM) and 50 µl RPMI+. Treated cultures received 25 µl cells, 25 µl RPMI+ with 0.1 µg PWM and 2.5, 5 or 10 µl of HTCM (n=3), I-HTCM (n=3) or I-CM (n=1) diluted in 50 µl RPMI+. All cultures were carried out simultaneously with the same 3 PBL populations and the final volume of each well was 100 µl. Cultures were maintained in an incubator at 37°C in a humidified atmosphere of 5% CO_2 and air. ^{3}H-thymidine was added to the culture (1µCi/well) 99 h

after the start of culture and cells were harvested 15 h later. Radioactivity on glass fiber filters was determined by liquid scintillation counting.

Radioimmunoassay. The radioimmunoassay (RIA) used to quantify PGE_2 in each HTCM and I-HTCM sample was conducted according to published procedures (Hwang et al., 1975; Hwang, 1985). ^3H-PGE_2 (100-200 Ci/mmol) used in the RIA was obtained from DuPont NEN Research Products (Boston, MA). An aliquot of HTCM from one conceptus was divided into two fractions using a 30,000 MW cut-off microconcentrator. The retentate fraction (>30,000 MW) was diluted back to its original volume with RPMI and the concentration of PGE_2 was determined. A sample of RPMI served as a control for any background interference that might be associated with the culture medium.

Statistical Analysis. Average cpm for quadruplicate wells of the lymphocyte proliferation assay were calculated and the results analyzed by ANOVA using the general linear model procedures of the SAS computer package (Luginbuhl et al., 1985). To determine immunosuppressive activity, lymphocyte proliferation in I-CM, HTCM and I-HTCM treated cultures was compared to that in stimulated control cultures using Tukey's mean comparison tests. Similarly, Tukey's mean comparison tests were used to determine within treatment differences between the addition of 2.5, 5 and 10 µl/well of I-CM, HTCM and I-HTCM.

RESULTS

Lymphocyte proliferation assay. The results of the lymphocyte proliferation assay are shown in Fig. 7. Each data point

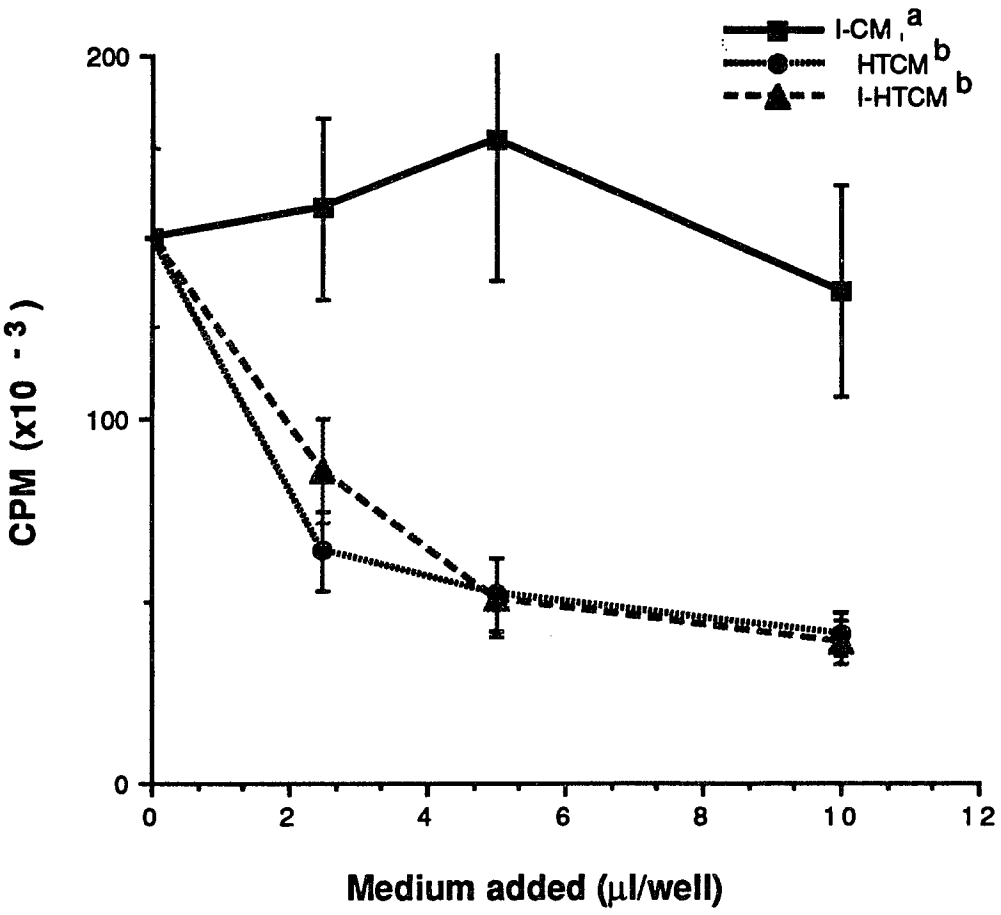

Figure 7. The effect of indomethacin control medium (I-CM), horse trophoblast-conditioned medium (HTCM) and indomethacin-treated HTCM (I-HTCM) on PWM-stimulated horse PBL proliferation. Different superscripts designate differences in lymphocyte proliferation ($P<.01$). Stimulated control values (150,000 ± 25,000 cpm; mean ± SEM) did not differ ($P>.05$) from those of I-CM-treated cultures. Lymphocyte proliferation was less ($P<.01$) in both HTCM and I-HTCM treated cultures as compared to that in stimulated controls.

represents the mean ± SEM (cpm) for three lymphocyte populations treated with I-CM (n=1), HTCM (n=3) and I-HTCM (n=3). PWM-stimulated lymphocyte proliferation in HTCM and I-HTCM treated cultures was less ($P<.01$) than that in I-CM-treated or stimulated control cultures. Proliferation in I-CM-treated cultures did not differ ($P>.05$) from that in stimulated control cultures. Immunosuppressive activity of HTCM and I-HTCM did not differ ($P>.05$). Results of the within treatment comparisons indicated no differences ($P>.05$) in proliferation with the different quantities of HTCM and I-CM. However, the addition of 10 µl of I-HTCM suppressed lymphocytes more ($P<.05$) than the addition of 2.5 µl I-HTCM.

Radioimmunoassay. The concentration of PGE_2 in each sample of HTCM and I-HTCM, as determined by the PGE_2 RIA, are shown in Table 4. The effectiveness of indomethacin, as a PGE_2 inhibitor in the trophoblast cultures, was determined by comparing the concentration of PGE_2 in I-HTCM to that in HTCM for each trophoblast sample. Addition of indomethacin to horse trophoblast cultures reduced PGE_2 production by 91 ± 1.6%. Not shown in Table 4 are the data for the >30,000 MW fraction of HTCM (Tissue sample 1) which was 38 pg/ml PGE_2.

DISCUSSION

The results of this study indicate that PGE_2 is not the primary factor responsible for horse trophoblast-derived immunosuppressive activity. Our results differ from what has been reported for decidual type placentae of humans and mice where PGE_2 appears to play a critical role in the suppression of immune cells at the fetal-maternal interface.

TABLE 4. PGE_2 concentrations (ng/ml) in horse trophoblast-conditioned medium (HTCM) and indomethacin-treated HTCM (I-HTCM)[a].

Trophoblast tissue sample[b]	HTCM	I-HTCM	% Decrease[c]
1	18.56	1.33	93
2	4.46	.50	89
3	15.02	1.42	91
Mean ± SEM	12.68 ± 7.4	1.08 ± .53	91 ± 1.6

[a]The RPMI control value was 0.00 pg/ml.

[b]Trophoblast was obtained from day 21 horse conceptuses. Tissue sections from each trophoblast sample were cultured in RPMI and RPMI + indomethacin to generate HTCM and I-HTCM, respectively.

[c]The inhibitory effect of indomethacin on PGE_2 production was calculated using the equation (1 - I-HTCM/HTCM x 100).

In the murine species, decidua contain T lymphocytes of several subsets (Kearns and Lala, 1985) as well as natural killer cells (Scodras et al., 1985). Excessive NK cell infiltration into the feto-placental unit appears to be responsible for spontaneous abortion of specific murine pregnancies (Gendron and Baines, 1988). During successful murine pregnancies, suppressor cells in the decidua and decidual cells themselves, inactivate NK cells (Croy et al., 1985; Gambel et al., 1985; Scodras et al., 1990). Recently, it has been shown that NK cell suppression is mediated by PGE_2, and the addition of indomethacin (10^{-5} M) or anti-PGE_2 antibody revives NK activity in the decidua (Scodras et al., 1990). Additional in vivo evidence concerning the significance of PGE_2 in maintaining pregnancy was provided by Lala et al. (1990), who demonstrated that chronic administration of indomethacin to pregnant mice causes abortion and embryo resorption.

Similar to murine placentae, first trimester human placentae contain decidual cells and decidual macrophages that secrete PGE_2 which, in turn, inhibits T lymphocyte activity by blocking expression of the interleukin-2 receptor and inhibiting the production of interleukin-2 (Lala et al., 1988). However, in agreement with our findings, Uren and Boyle (1990) demonstrated that suppressive activity of human placental macrophages was not inhibited by indomethacin. Additionally, Scodras et al. (1990) found that the concentration of PGE_2 in murine decidual cell supernatants following 20-hr in vitro culture was much lower than that required for NK cell suppression induced by decidual cells. The authors suggested that, in addition to PGE_2, decidual cells produce other suppressor molecules. Our data

support this theory. While indomethacin effectively decreased PGE_2 production, immunosuppressive activity of HTCM and I-HTCM did not differ, thus it appears there are factors other than PGE_2 responsible for the immunosuppressive activity in HTCM. These results are not surprising in view of the fact that conceptus-derived soluble suppressor proteins have been identified in a number of species (Clark et al., 1986; Daya et al., 1986; Murray et al., 1987).

There are three additional pieces of evidence supporting our conclusion that PGE_2 is not responsible for HTCM-induced immunosuppression. First, PGE_2 is capable of inhibiting ^3H-thymidine incorporation by mitogen-stimulated lymphocytes at concentrations of 10^{-8} M, but to attain at least 65% suppression, a concentration of 10^{-7} M is required (Goodwin et al., 1977). In this study, we achieved 65% suppression of lymphocyte proliferation with PGE_2 concentrations in the lymphocyte culture wells ranging from 10^{-9} to 10^{-11} M. Furthermore, it has been reported that PGE_2 does not inhibit PWM-stimulated peripheral blood lymphocytes (Goodwin et al., 1977). Yet, in this study and in previous work (Roth et al., 1990), we have demonstrated potent suppression of PWM-stimulated horse PBLs with horse conceptus-conditioned medium. Finally, we previously reported that following amicon microconcentrator centrifugation, the immunosuppressive activity of horse conceptus-conditioned medium is contained in the >30,000 MW retentate fraction (Roth et al., 1990). While in that report we mentioned the apparent lack of hormone or prostaglandin contribution to the suppressive activity, we could not rule out the possibility that these substances remained in the >30,000 MW fraction. In this study, we determined that a single passage

through the microconcentrator reduced the PGE_2 concentration of the retentate fraction by 99.7% relative to that of the whole sample.

Together, data from previous work indicating the large size of the horse conceptus-derived suppressor factor(s) (Roth et al., 1990) and from this study in which the inhibition of prostaglandin production did not affect suppressor activity of cultured horse trophoblast, indicate that PGE_2 is not the primary immunosuppressant produced by the pre-implantation horse conceptus.

CHAPTER V

HORSE CONCEPTUS-DERIVED SUPPRESSION OF T AND B LYMPHOCYTE PROLIFERATION: INTERLEUKIN-2 RECEPTOR INVOLVEMENT[4]

INTRODUCTION

The immunosuppressive activity of a >30,000 molecular weight (MW) factor present in horse conceptus conditioned medium (HCCM) has previously been reported (Roth et al., 1990). Results from subsequent work have shown this suppressor factor (HCCM-SF) to be >100,000 MW. High molecular weight components of conceptus cultures and/or gravid uterine fluid of other domestic species, including the cow (Segerson and Bazer, 1989), sheep (Hansen et al., 1987; Murray et al., 1987) and pig (Murray et al., 1987), also suppress lymphocytes when supplemented to in vitro blastogenesis cultures. It has repeatedly been suggested that these suppressive factors play a role in protecting the conceptus from maternal immunological attack during pregnancy. In the horse, one such role may be to temporarily protect the endometrial cups which, although surrounded by lymphocytes, are not destroyed until day 120 of pregnancy (Allen, 1979). Thus far, most investigations have been primarily directed towards identifying and characterizing uterine and conceptus-derived factors (Murray et al., 1978; Godkin et al., 1982; Hansen et al, 1987; Newton et al., 1988; McDowell et al., 1990) while their functional significance remains largely unknown.

Some information pertaining to the immunosuppressive mechanisms of components obtained from uterine fluid or conceptus tissue has been

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[4] Roth, T.L., K.L. White, D.L. Thompson, Jr., S. Rahmanian and D.W. Horohov. J. Reprod. Fertil. (In review).

reported for humans (Pockley and Bolton, 1990; Saito et al., 1990), mice (Clark et al., 1985; Mayumi et al., 1985; Clark et al., 1986), sheep (Segerson, 1988) and, more recently, cows (Segerson and Libby, 1990). In these studies, suppressor activity was identified in lymphocyte cultures as decreased ^{3}H-thymidine uptake by cells following mitogen stimulation. There are mitogens available that stimulate B and/or T lymphocytes preferentially (Sharon, 1983), but in most immunosuppressive studies only PHA, a potent T cell activator, has been used to stimulate lymphocyte proliferation. Because cell-mediated and not humoral immunity is of greater concern in fetal allograft acceptance, T cells have been the focus of most attempts to elucidate suppressor factor mechanisms.

Production of IL-2 and the expression of the high affinity IL-2R are critical for proliferation of T cells in response to either PHA or antigen (Depper et al., 1984). The IL-2R has been identified and described as a dimer consisting of a p55 alpha chain with low affinity for IL-2 and a p75 beta chain with slightly greater IL-2 affinity (Greene et al., 1986; Tsudo et al., 1986; Teshigawara et al., 1987; Smith, 1988). With mitogenic or allogeneic stimulation, the two chains are expressed together on the cell surface to form the high affinity IL-2R complex which, when bound to IL-2, is largely responsible for signaling cell proliferation (Sharon et al., 1986; Smith, 1988). Recombinant human IL-2 (rIL-2) will interact with the IL-2R of many species, including the horse (Fenwick et al., 1988). An antibody to the IL-2R (anti-Tac) blocks IL-2 binding (Robb et al., 1981; Leonard et al., 1982; Sharon et al., 1986), inhibits cell proliferation (Robb et al., 1981) and may even prevent allograft rejection (Kirkman et

al., 1985). Data have suggested that uterine/conceptus suppressor factors act similarly, disrupting the IL-2/IL-2R interaction to inhibit blastogenesis. A soluble suppressor factor obtained from mouse decidua blocks the response of T cells to IL-2 by interfering with IL-2R (Clark et al., 1985; Clark et al., 1986). Pregnancy zone protein reduces IL-2 production but not IL-2R expression (Saito et al., 1990). Human placental supernatant inhibits both IL-2 and IL-4 driven proliferation of CTLL-2 cells (Menu and Chaouat, 1989), an IL-2 dependent murine cell line (Gillis et al., 1978). Suppressive activity of substances found in uterine luminal fluid of pregnant sheep and cows may also be mediated through alterations of the IL-2 system. In both species, uterine fluid suppressor factors block IL-2 mediated blastogenesis of T cells (Segerson, 1988; Segerson and Gunsett, 1990), and it has been suggested that bovine uterine luminal proteins act by blocking IL-2R recognition in addition to binding directly to IL-2 (Segerson and Libby, 1990).

While mitogen-stimulated lymphocyte proliferation assays have provided valuable information about the in vitro response of these cells to immunosuppressive factors, the response of specific cell subsets has not been determined. Antibodies to unique cell subset antigens (CD4, CD5 and CD8) are available for labeling specific cell populations. Human T cells express many cell surface molecules such as the CD5 molecule which is present on virtually all T cells (Hedrick et al., 1984). The T cell population can be divided into two broad subsets, T helper and T suppressor/cytotoxic cells, by their expression of CD4 and CD8 antigens, respectively (Sprent, 1989). Similarly, there exist antibodies to equine lymphocytes that bind

either the entire T cell population or a T suppressor cell subset (Wyatt et al., 1988). Specific fluorescent antibody labeling followed by fluorescence activated cell sorter (FACS) analysis provides an accurate method for immunophenotypically distinguishing and quantifying cell subsets in a mixed cell population (Jackson and Warner, 1986).

The experiments described herein were designed 1) to examine the affect of HCCM-SF on B and T lymphocytes using various mitogens and FACS analysis 2) to determine the temporal relationship of HCCM-SF activity and lymphocyte activation and 3) to better define the proposed IL-2 associated mechanism of lymphocyte suppression. The results of these experiments may increase our understanding of the fetal-maternal immunological interaction in utero.

MATERIALS AND METHODS

Animals and Conceptus Cultures. The horse breeding regimen and conceptus collection techniques were carried out as described in Chapter III. Conceptuses were collected by non-surgical uterine flushing on Day 20 (ovulation = Day 0) using sterile PBS supplemented either with 1% calf serum and 1% antibiotic/antimycotic (Group I), or with only 1% antibiotic/antimycotic (Group II). Under sterile conditions, conceptuses were rinsed twice in their respective medium, after which Group I conceptuses were rinsed once in RPMI+, and Group II conceptuses were rinsed in RPMI-1640 with 1% antibiotic/antimycotic (RPMI). The three Group I conceptuses were each placed in a 50-ml tissue culture flask containing 20 ml RPMI+. Cultures were incubated for 48 h at 37°C in a humidified atmosphere of 5% CO_2 and air. The three Group II conceptuses were each placed in a 50-ml tissue culture

flask containing 15 ml RPMI and were incubated for 30 h under the same conditions described for Group I. Within both Group I and Group II, conceptus culture supernatants (HCCM I and HCCM II, respectively) were pooled. Both pooled samples were centrifuged (600 x g; 20 min), filtered (.2 µm) and stored in aliquots at -80°C. A lymphocyte proliferation assay was used to determine that there was no difference ($p>.05$) in suppressive activity between HCCM I and HCCM II (Table 5). Control medium (CM) consisted of RPMI or RPMI+ incubated and stored similarly.

For some experiments, HCCM and CM samples were both fractionated by molecular weight using microconcentrator centrifugation. Two microconcentrators with 100,000 MW exclusion membranes were rinsed with 500 µl of RPMI-1640 (850 x g; 50 min). After rinsing, 500 µl of CM and HCCM were each pipetted into one of the microconcentrators. Samples were centrifuged (850 x g; 40 min) and flow-through fractions (<100,000 MW) were collected. Retentate fractions were resuspended in 500 µl RPMI-1640 and, again, centrifuged. Flow-through volumes were discarded. Retentate was resuspended to 500 µl, the centricons were inverted and the retentate fractions (>100,000) were collected off the filter membranes into the collection cups by centrifugation (600 x g; 5 min). Both fractions of CM and HCCM were filtered (.2 µm) and diluted to 600 µl/ml in RPMI+.

Lymphocyte Proliferation Assays. Unless otherwise stated, the lymphocyte proliferation assays were carried out as follows. Lymphocytes were obtained according to procedures described in Chapter III. Cultures were prepared in quadruplicate for each PBL population in 96-well round-bottomed tissue culture-treated plates and each well

TABLE 5. The effect of HCCM I and HCCM II on PWM-stimulated lymphocytes.[a]

Volume added (µl/well)	HCCM I	HCCM II
0 (control)	44 ± 8[b]	41 ± 7[b]
1	36 ± 4[c]	32 ± 4[c]
5	22 ± 3[cd]	20 ± 1[cd]
10	15 ± 2[e]	12 ± 1[e]

[a]Values represent mean cpm ± SEM (x 10^{-3}) for three lymphocyte populations.

[bcde]Different superscripts designate differences ($p<.05$; Tukey mean comparisons).

received 1 x 10^5 cells (25 µl). PWM, diluted in RPMI-1640, was added to stimulate cell proliferation (.1 µl PWM/well). HCCM or fractionated HCCM was added in concentrations specified for each assay, and final well volume was 100 µl. The assays were completed as described in Chapter III.

Preliminary Assay. To determine the concentrations of PHA required to induce optimal and sub-optimal proliferative responses in our lymphocyte assay, three.populations of PBLs were tested with serial dilutions of PHA (3 to .0625 µl/well). Maximal cell proliferation was achieved using 1 µl/well while a suboptimal response (approximately 50% of maximum) resulted from the use of .125 µl/well (Fig. 8).

Mitogen Comparison Assay. The suppressive activity of HCCM II on LPS-, PHA- and PWM-stimulated lymphocytes was compared. HCCM II was serially diluted in RPMI+ such that treated wells contained HCCM II at 20 to 1.25 % of total volume while control wells received only RPMI+. PWM (4 µg/ml), PHA (5 µl/ml) and LPS (.4 µg/ml) were added to wells in 25 µl volumes. In our lab, these concentrations of LPS and PWM stimulate maximal proliferation of equine lymphocytes. PHA was added at a sub-optimal dose, one found to stimulate proliferation at approximately 50% of maximum, in order to increase the sensitivity of the PHA-treated cells to HCCM suppression. The three mitogen assays were run simultaneously with PBLs from three randomly chosen mares. PHA- and PWM-stimulated cultures were pulsed with ^{3}H-thymidine at 80 h and harvested at 96 h. LPS-stimulated cultures were pulsed at 96 h and harvested at 112 h since we found LPS-treated cells require more time to achieve their maximal proliferative response.

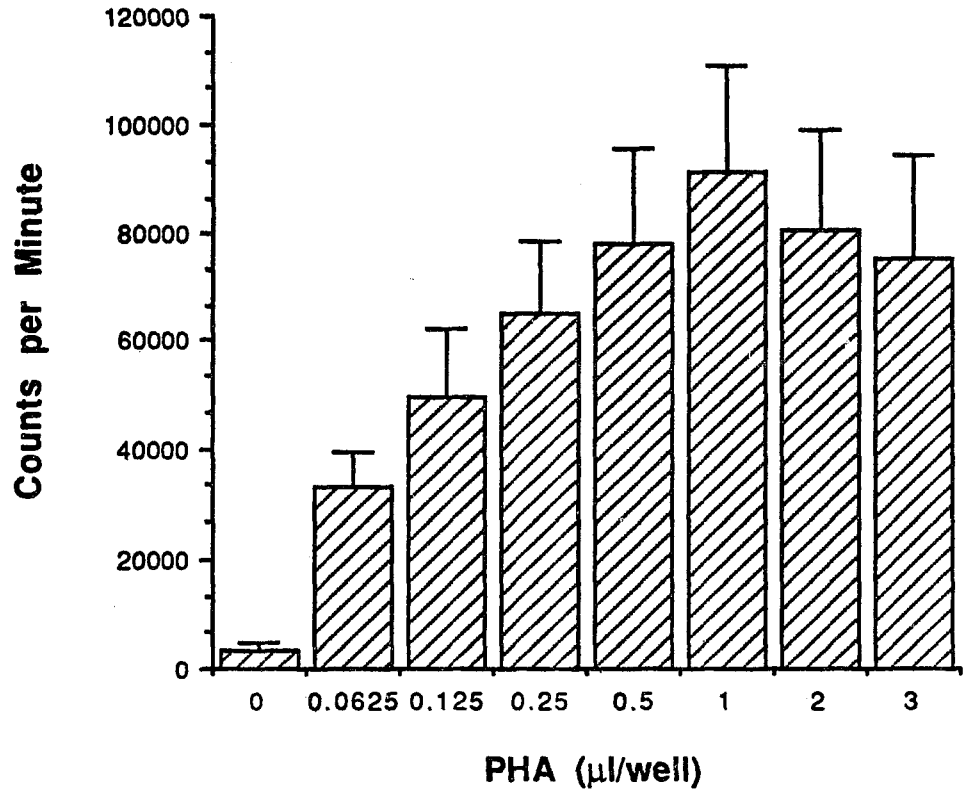

Figure 8. Serial dilutions of PHA were tested in an equine lymphocyte proliferation assay to determine the PHA concentrations required to induce optimal and sub-optimal proliferative responses in our assay system. Data represent average cpm ± SEM for three equine lymphocyte populations.

Temporal Effect of HCCM. To determine the stage of cell activation with which HCCM-SF interferes, HCCM I was supplemented to lymphocyte cultures 0, 24 and 48 h following PWM stimulation of cells. The lymphocyte proliferation assay was prepared as previously described using lymphocytes from three randomly chosen mares. On the 96-well plate, triplicate rows were designated, Time 0, Time 24 and Time 48. HCCM I was serially diluted in RPMI+ to three final concentrations of 480, 240 and 120 µl/ml. Each of the Time 0 rows were supplemented with a different dilution of HCCM I (25 µl/well). After 24 h incubation, each of the three Time 24 rows received 25 µl/well of the same three HCCM I dilutions. At 48 h the three Time 48 rows were similarly supplemented. At each treatment period, those wells not receiving HCCM I received 25 µl RPMI+ such that final volume in all wells was 150 µl. PWM was supplemented to all but non-stimulated control wells at Time 0. All wells were pulsed at 100 h and harvested at 116 h. This experiment was carried out twice with a total of six PBL populations, and data from both experiments were pooled for statistical analysis.

Cells were treated with HCCM II prior to mitogen stimulation to determine if HCCM-treated lymphocytes are permanently suppressed. PBLs from two mares were prepared as described for the lymphocyte proliferation assays. The pre-stimulated cultures consisted of four treatments set up in sterile cryovials as follows: 1) Non-stimulated - 150 µl cells + 450 µl RPMI+ 2) Control - 300 µl cells + 900 µl RPMI+ 3) HCCM 10 - 120 µl HCCM II + 300 µl cells + 780 µl RPMI+ and 4) HCCM 5 - 60 µl HCCM II + 300 µl cells + 840 µl RPMI+. Vials were vortexed and incubated with loosened caps for 24 h. At the end of

incubation, cells were washed twice in RPMI+ (400 x g; 10 min) and resuspended at 2 x 10^6 cells/ml. Cells from each pre-stimulated culture were pipetted into wells (50 µl/well) and were treated with 25 µl RPMI+ containing 0 µl (pre-treated only cells), 5 µl (pre/post-treated 5) or 10 µl (pre/post-treated 10) HCCM II. PWM was added to all but non-stimulated control wells at this time. Assay was completed as previously described.

Equine Lymphocyte Supernatant Supplementation. Equine PBMC supernatant (PBMCS) was generated by combining PBMCs from three mares, diluting cells in RPMI+ to a concentration of 5 x 10^6/ml (10 ml total) in a tissue culture flask and adding 150 µl PHA. After 6 h incubation, the cell suspension was pipetted into a 15 ml centrifuge tube and centrifuged (400 x g; 10 min). Medium was discarded and cells resuspended in 10 ml fresh RPMI+. Cultures were then incubated for 18 h, cells were again centrifuged (600 x g; 10 min) and PBMCS was obtained. Control medium consisted of 10 ml of the same RPMI+ incubated for 18 h in a tissue culture flask. The PBMCS assay was prepared with three lymphocyte populations. Two 96-well plates were set-up with serial dilutions of HCCM II (10 to .625 µl/well). Cells were added to wells (1 x 10^5 /well), and PWM was added to all but non-stimulated control wells. All wells of one plate received 50 µl PBMCS while all wells of the second plate received 50 µl CM. After 24 h of culture, wells received another 40 µl of their respective medium bringing total well volume to 150 µl. The assay was completed as previously described.

IL-2 Supplementation. The effect of supplementing HCCM-treated equine lymphocytes with recombinant human interleukin-2 (rIL-

2; Cetus Corp., Emeryville, CA) was examined in two assays designed to determine if supplementation of rIL-2 to HCCM-treated lymphocytes would override the suppressive effect of HCCM. PBLs from three mares were used. In the first experiment, two sets of quadruplicate wells of each PBL population were supplemented with 3, 6, 12 and 24 µl of HCCM I. One set of each quadruplicate pair also received rIL-2 (200 U/ml). PHA (.125 µl/well) was added to all wells, cells were pulsed at 80 h and harvested at 96 h.

In the second experiment, HCCM II was diluted in RPMI+ to 600 µl/ml and supplemented to cultures in 25 µl volumes. Cell proliferation was stimulated with a sub-optimal dose of PHA (.125 µl/well). Serial dilutions of rIL-2 (50 to .78 U/ml) were also supplemented to cultures. To serve as a negative control, non-stimulated cells were similarly supplemented with rIL-2. The positive control consisted of cells, PHA and rIL-2, but no HCCM. Cultures were pulsed at 80 h and harvested at 96 h.

CTLL-2 Cells. Unlike resting lymphocytes, CTLL-2 cells continuously express the high affinity IL-2R complex, and the addition of small concentrations of IL-2 will immediately stimulate these cells to proliferate, while a deficiency of IL-2 will lead to their rapid demise (Gillis, 1978). To better understand the effect of HCCM on IL-2R function, fractionated HCCM II was added to CTLL-2 cell cultures. Triplicate wells of a 96-well plate were prepared with seven serial dilutions of rIL-2 (50 to .78 U/ml) to serve as a standard. In each of the following rows, rIL-2 was added to triplicate wells at concentrations identical to those in dilutions 3-6 of the standard (12.5 to 1.56 U/ml). Each of these rows also received 50 µl/well of

one of the two diluted fractions of HCCM II or CM. The standard wells received 50 µl RPMI+. CTLL-2 cells were added to all wells (2,000 cells in 50 µl) and cultures were incubated for 24 h before cells were pulsed with ^{3}H-thymidine (1 µCi/well). Cultures were harvested 4 h after pulsing and radioactivity was determined by liquid scintillation counting.

Murine Splenocytes. Mouse splenocytes were obtained from spleen homogenate by Ficoll-gradient centrifugation and interface cells were recovered. Cells were rinsed twice with HBSS and resuspended in RPMI+ at 7 x 10^{6} cells/ml. Splenocytes were added to two 96-well plates in 25 µl volumes and were stimulated by the addition of 50 µl of PWM, PHA or Con A (5, 2.5 and 5 µg/ml, respectively). Fractionated HCCM II was supplemented to treated wells of one plate (20 µl/well), while stimulated control wells received 20 µl of each CM fraction. Cells were pulsed at 88 h and harvested at 100 h.

FACS Analysis. FACS analysis was used to determine the effect of HCCM II on proliferation of specific lymphocyte subsets. HCCM-treated cell populations were compared to non-treated cells using three antibodies to different equine lymphocyte markers. Antibodies were, the monoclonal antibodies (mAb) HT23A and HT14A (pan T and T suppressor cell subset antibodies, respectively; VMRD, Pullman, WA), and an FITC-conjugated polyclonal goat anti-equine IgG antibody (B cell antibody). The conjugate for the mAbs was an FITC-conjugated goat anti-mouse IgG antibody (VMRD, Pullman, WA). Two equine PBL populations were analyzed. For each population, five tissue culture flasks were prepared as follows: 1) Control - 4 ml RPMI+ 2) PHA -

6.25 µl PHA + 4 ml RPMI+ 3) PWM - 20 µl PWM + 4 ml RPMI+ 4) PHA+ - 6.25 µl PHA + 500 µl HCCM II + 3.5 ml RPMI+ 5) PWM+ - 20 µl PWM + 500 µl HCCM II + 3.5 ml RPMI+. To each flask, 1 ml of cells suspended in RPMI+ at 5 x 10^6 cells/ml was added. Flasks were incubated for 96 h and, using a hemacytometer, cell concentration was determined at the end of culture. The cell suspension in each flask was divided into five sterile tubes (1 ml/tube). Tubes were labeled as: autofluorescent control, FITC control, mAb HT23A, mAb HT14A and IgG-FITC. Cells were pelleted (400 x g; 10 min) and medium was decanted. Cells were rinsed with 3 ml volumes of PBS and decanted again. The monoclonal antibodies, HT23A and HT14A, were diluted 1:1000 in PBS supplemented with 10% goat serum (PBS-GS). The polyclonal goat anti-equine IgG-FITC and goat anti-mouse IgG-FITC antibodies were diluted 1:400 in PBS-GS. Diluted antibodies were added to appropriate tubes (50 µl/tube) while autofluorescent and FITC controls received 50 µl cold PBS-GS. Tubes were vortexed and incubated on ice for 1 h. All samples were washed with 3 ml volumes of ice cold PBS-GS. After decanting, 75 µl cold 1% p-formaldehyde was added to IgG-FITC tubes to fix cells. Tubes were vortexed and stored at 4°C. All remaining tubes, except the autofluorescent controls, received 50 µl of diluted goat anti-mouse IgG-FITC. Autofluorescent controls received 50 µl PBS-GS. After vortexing, cells were incubated on ice 1 h, then washed and fixed as described for cells in IgG-FITC tubes. Samples were stored at 4°C for 48 h then diluted with 100 µl PBS-EDTA and analyzed on a FACS 440 (Becton Dickinson, Mountain View, CA). For each sample, 10,000 cells were analyzed for both fluorescence and light scatter using a Consort 40 analyzer (Becton Dickinson, Mountain View, CA). Gate

settings were determined based on autofluorescent and FITC controls to eliminate any non-specific and/or background fluorescence. Due to between-flask variation in cell concentration following the 96 h culture, data were recorded herein as average cell number/ml (final cell concentration in flask x % of 10,000 labeled) such that results for a specific cell population would not be biased by a change in the size of other cell subset populations. The two-dimensional scatter analysis was used to compare blast cell populations between treatments. The percent blast cells was determined by comparing stimulated to non-stimulated FITC control samples. Gates were set such that the windowed area contained the larger cells present only in the stimulated samples. A window with the same gate settings was then applied to the HCCM-treated, stimulated samples, and the percentage of cells (blast cells) in this area was recorded.

Statistical Analysis. Average cpm and percent of stimulated control values were calculated for triplicate or quadruplicate wells of proliferation assays. Statistical analysis was performed using data recorded as cpm if the stimulated control values were the same for the treatments being tested. If the stimulated control values differed between treatment groups, data were expressed and analyzed as % of stimulated control. Results, in the appropriate form for each assay, were analyzed in a factorial arrangement by ANOVA using the general linear model procedures of the SAS computer package (Luginbuhl et al., 1985). All main effect interactions were tested (i.e. treatment or time x HCCM or rIL-2 dose). Between treatment groups and within treatment differences were determined by Tukey mean comparison tests. To further compare the effect of rIL-2 on HCCM-treated cells to that

on non-treated cells, regression analysis was carried out with data in each of the two rIL-2 supplementation assays. Treatment curves were compared and tested for differences in intercepts, linear functions and quadratic functions, and insignificant terms were dropped from the model.

RESULTS

Mitogen Comparison. The effect of HCCM-SF on lymphocyte cultures differed ($p<.01$) depending on the mitogen used to stimulate lymphocyte proliferation (Table 6). The results of a 3 x 6 factorial arrangement ANOVA and Tukey mean comparisons showed that, compared to stimulated controls, the percent suppression of HCCM-treated cell proliferation was greater ($p<.01$) in PWM-stimulated cultures than in LPS- and PHA-stimulated cultures. PHA-stimulated cells were suppressed ($p<.01$) by high concentrations of HCCM (10 and 20 µl/well), but LPS-stimulated cells were unaffected ($p>.05$) at all levels of HCCM (within mitogen Tukey mean comparisons).

Temporal Effect of HCCM. Suppressive activity of HCCM-SF decreased ($p<.001$) if HCCM I was supplemented to lymphocyte cultures after the start of stimulation (Fig. 9). Although still suppressive, HCCM added to cultures 24 h after the start of stimulation was less ($p<.01$) effective then if added at Time 0. By 48 h following cell stimulation, the addition of HCCM to the cultures resulted in only a slight decrease in proliferation, much less ($p<.01$) than that observed when HCCM was added at Time 24. There were no main effect interactions ($p>.05$).

Lymphocytes pre-treated with HCCM II exhibited the capacity to respond maximally to mitogen stimulation (Fig. 10). Proliferation of

TABLE 6. Effect of HCCM II supplementation to mitogen-stimulated lymphocytes[a].

	Mitogen		
	LPS[b]	PHA[c]	PWM[d]
Non-Stimulated	8,990 ± 322	5,657 ± 578	14,712 ± 843
HCCM (µl/well)			
0 (Control)	21,184 ± 2,713	76,648 ± 18,131	77,111 ± 19,572
1.25	20,180 ± 3,553	72,660 ± 17,789	63,138 ± 13,418*
2.5	19,446 ± 788	72,762 ± 16,364	43,365 ± 9,260*
5	18,799 ± 1,420	68,037 ± 14,010	30,595 ± 5,274*
10	20,700 ± 1,117	58,726 ± 13,552*	22,271 ± 2,677*
20	19,187 ± 682	48,905 ± 11,160*	12,155 ± 1,094*

[a]Values represent mean cpm ± SEM for three lymphocyte populations.

[bcd]Different superscripts designate between mitogen differences in overall suppressor activity ($p<.01$) as determined by Tukey mean comparisons using data expressed as % of control. There was a significant mitogen x HCCM interaction ($p<.01$).

*Differs from control ($p<.01$); within mitogen Tukey mean comparisons.

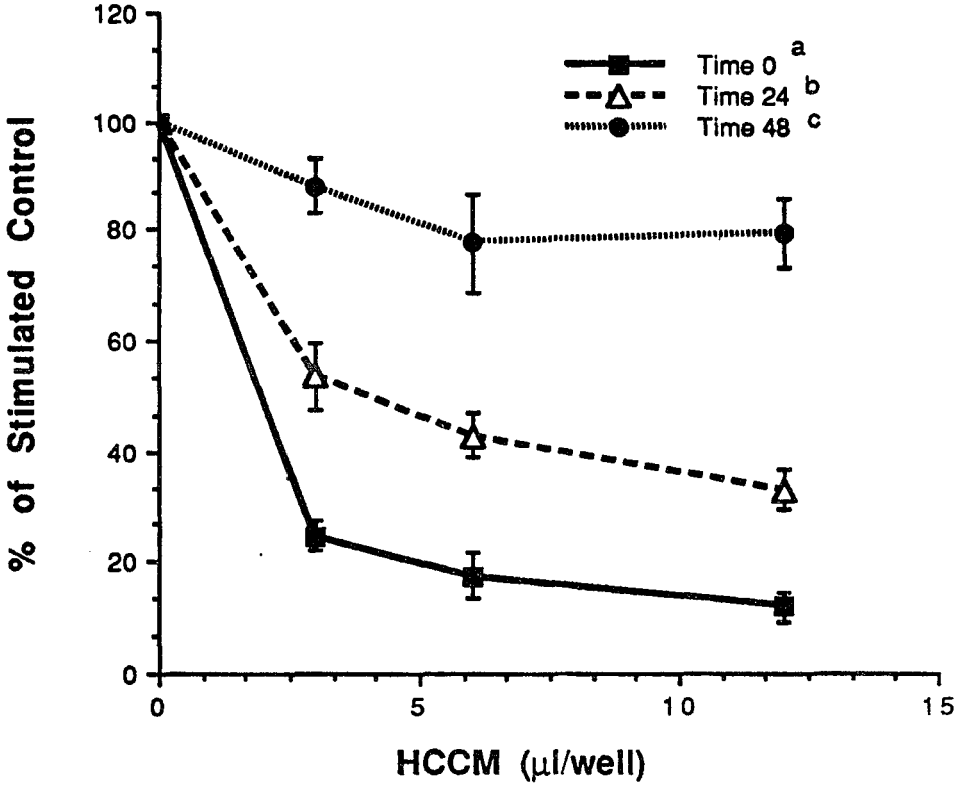

Figure 9. Effect of HCCM I supplementation to PWM-stimulated lymphocyte cultures at 0, 24 and 48 hours after the start of culture. Values represent mean % of stimulated control ± SEM for between lymphocyte population variation (n=6). Different superscripts denote an overall difference in suppression between times ($p<.01$; Tukey mean comparisons). There were no significant interactions ($p>.05$).

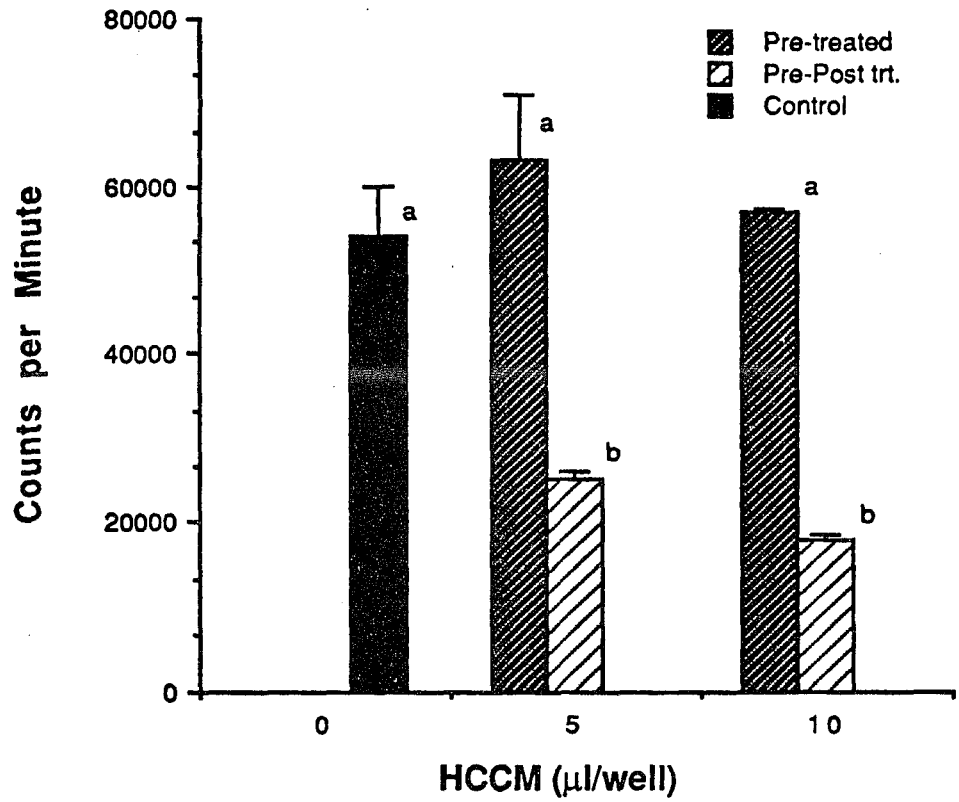

Figure 10. Response to PWM-stimulation following pre-incubation of cells with HCCM II. Values represent mean cpm ± SEM for two lymphocyte populations. Compared to the stimulated control (no HCCM), there was no difference ($p>.05$) in the proliferative responses of HCCM I pre-treated cells, while the responses of pre/post-treated cells were suppressed ($p<.01$). Different superscripts denote differences in ^{3}H-thymidine incorporation ($p<.01$; Tukey mean comparisons).

cultures pre-treated with 5 or 10 µl HCCM/well did not differ ($p>.05$) from that of the stimulated control. However, the proliferative response of pre-treated cells to which 5 and 10 µl/well HCCM was again added during mitogen stimulation (pre/post-treated), was much less ($p<.01$) than that of both the pre-treated only cells and the stimulated control.

Equine Cell Supernatant and IL-2 Supplementation. PHA-stimulated PBMC supernatant was added to HCCM II-treated equine lymphocytes in an attempt to augment their proliferative response to PWM (Fig. 11), but compared to proliferation in control medium-supplemented cultures, there was no difference ($p>.05$). The addition of an extremely high concentration of rIL-2 (200 U/ml) to HCCM I-treated cells did not override the suppressive activity of HCCM I (Fig. 12). Although proliferation in cultures supplemented with rIL-2 was greater ($p<.01$) than that in cultures without rIL-2, a dose-dependent decrease in proliferation with the addition of HCCM was observed even in cultures with 200 U/ml of rIL-2 ($p<.01$). Furthermore, results from the regression analysis showed that both treatment curves were linear with the same slope ($p>.05$) and only the intercepts were different ($p<.01$). Similarly, in Figure 13, regression analysis was used to determine that the slopes of the two treatment curves, PHA and PHA + HCCM, were linear and did not differ ($p>.05$). Again, only the intercepts were different ($p<.01$). The results of the 3 x 7 factorial ANOVA and Tukey mean comparisons showed that overall proliferation in HCCM II-treated cultures was less ($p<.01$) than that in PHA control cultures, but greater ($p<.01$) than that in non-stimulated cultures.

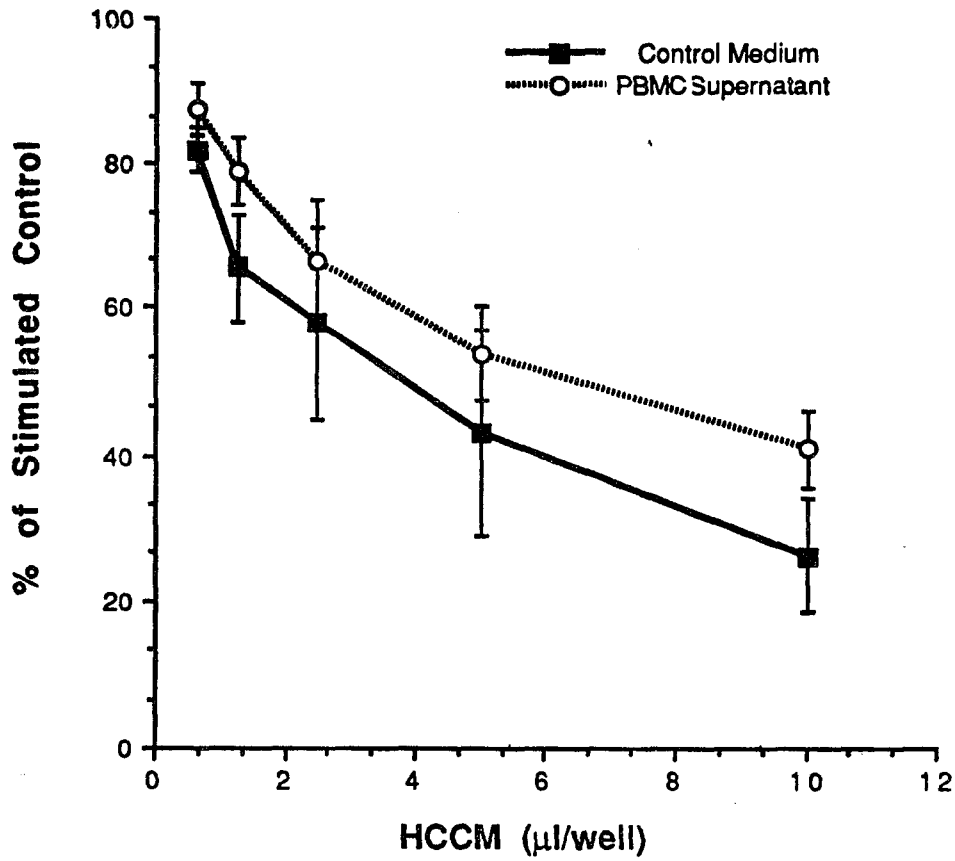

Figure 11. Effect of PHA-stimulated equine PBMC supernatant supplementation to HCCM II-treated, PWM-stimulated lymphocytes. Data represent mean % of control ± SEM for between lymphocyte population variation (n=3). Proliferation of cells supplemented with equine PBL supernatant did not differ ($p>.05$) from that of cells receiving control medium.

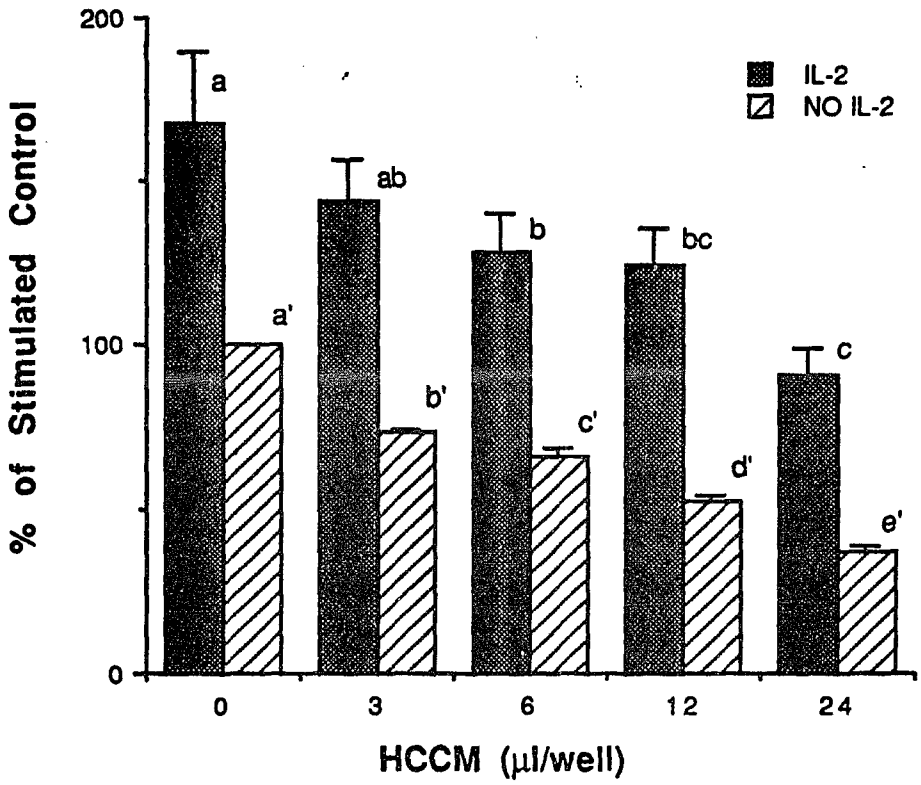

Figure 12. Effect of human recombinant IL-2 supplementation (200 U/ml) on proliferation of HCCM I-treated, PHA-stimulated equine PBLs. Data represent mean % of control (no HCCM or rIL-2 added) ± SEM for between lymphocyte population variation (n=3). In both rIL-2 supplemented and non-supplemented cultures, cell proliferation decreased ($p<.01$) with the addition of HCCM I. Different superscripts designate within treatment differences (a-c and a'-e' for within IL-2 and no IL-2 treatments, respectively; $p<.01$; Tukey). There was no treatment x HCCM interaction ($p>.05$). Although the intercepts of the two treatment curves were different ($p<.01$), the linear slopes of the two curves did not differ ($p>.05$; ANOR; $R^2=.84$).

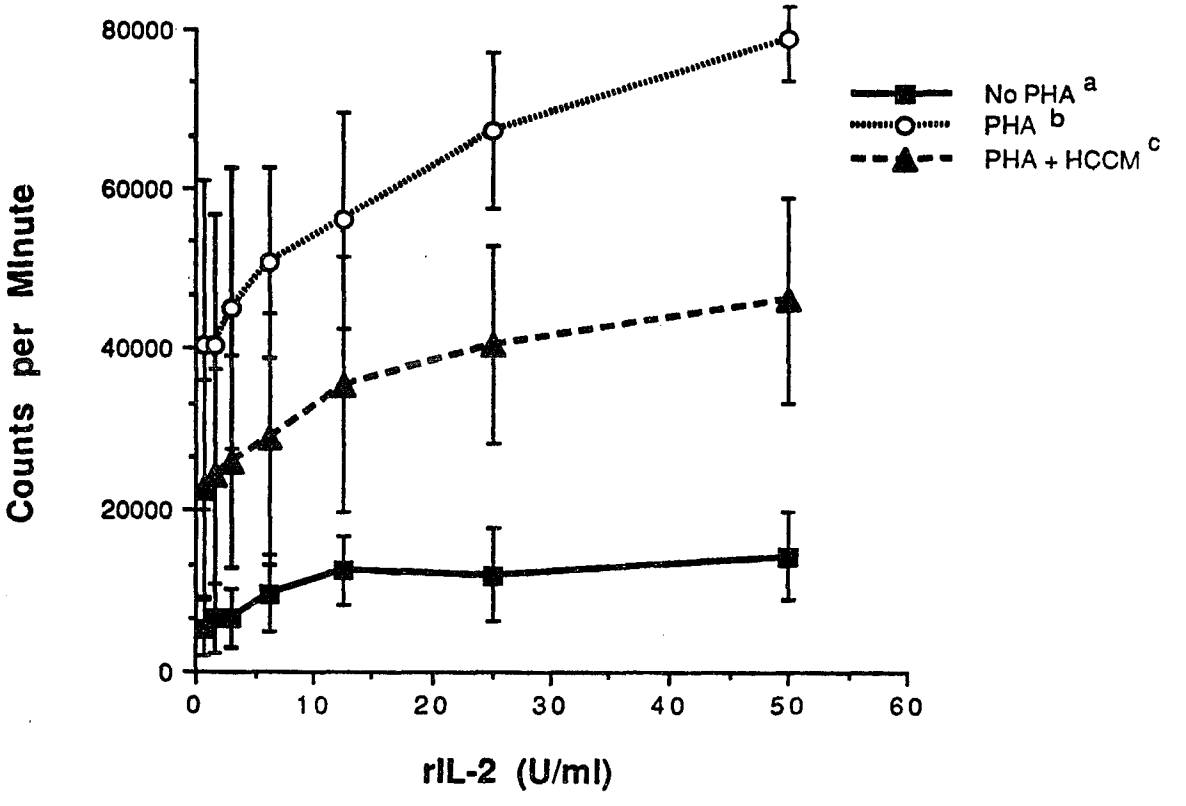

Figure 13. Effect of rIL-2 supplementation to non-stimulated, PHA-stimulated and PHA-stimulated + HCCM II-treated cell cultures. Data represent mean cpm $\pm$ SEM for between lymphocyte population variation (n=3). rIL-2 supplementation increased ($p<.01$) proliferation of cells within each treatment. Different superscripts denote between treatment differences ($p<.01$) in overall ^{3}H-thymidine incorporation as determined by Tukey mean comparisons. While the intercepts of all three treatment curves differ ($p<.01$), the linear slopes of the PHA and PHA + HCCM curves are the same ($p>.05$; ANOR; $R^2=.73$).

CTLL-2 Cells and Murine Splenocytes. CTLL-2 cell proliferation in response to exogenous rIL-2 was not affected by the addition of the >100,000 MW fraction of HCCM, or the two fractions of CM (Table 7). However, the <100,000 MW fraction of HCCM did inhibit CTLL-2 proliferation ($p<.01$). While there was no treatment x rIL-2 interaction ($p>.05$), only in the standard did a decrease in rIL-2 result in a significant decrease in cell proliferation ($p<.05$). HCCM-SF appeared to be effective at suppressing PWM-stimulated murine splenocytes as incorporation of ^{3}H-thymidine was less ($p<.01$) in cultures treated with the >100,000 MW HCCM fraction than that in cultures treated with the > 100,000 MW CM fraction. However, there were no differences in proliferation between treatments within ConA or PHA ($p>.05$).

FACS Analysis. Data from the FACS analysis are recorded in Table 8. Because of the 96 h culture period prior to cell labeling and fixing, the cell concentration in each flask differed from the initial 1×10^{6}/ml, with there being a decrease in cell number in the control flask and an increase in the mitogen-stimulated flasks. For this reason, the data are presented as final cell numbers/ml. Relative to non-stimulated controls, there was an increase in total T cells with both PHA and PWM stimulation (+36,000 and +11,000 cells/ml, respectively). However, a more exaggerated increase in B cells was observed with both mitogens (+238,000 and +236,000 cells/ml, respectively). The addition of HCCM to PHA-stimulated cells resulted in a decrease of 66,000 T and 20,000 B cells/ml when compared to PHA-stimulated cells without HCCM. When HCCM was supplemented to PWM-stimulated cells, again, a decrease in T cells occurred (-19,000

TABLE 7. Effect of fractionated HCCM II and CM on CTLL-2 cells and murine splenocytes.

		HCCM[a]		CM[a]	
	Standard	<100[c]	>100[d]	<100[d]	>100[d]
CTLL-2 cells[b]					
rL-2 (U/ml)					
12.5	13.41 ± .45	6.28 ± 1.08	13.44 ± 1.52	10.45 ± .96	11.55 ± .17
6.25	13.48 ± .66	6.69 ± .19	13.68 ± .69	13.03 ± .51	13.89 ± .68
3.125	13.42 ± .51	6.49 ± .42	13.38 ± .77	10.34 ± .88	12.23 ± .82
1.56	10.70 ± .48*	5.80 ± 1.14	11.21 ± .98	9.05 ± .86	11.98 ± .18
Murine splenocytes[e]					
Mitogen		<100	>100	<100	>100
PWM		28.20 ± 2.03	2.79 ± .52**	32.67 ± 4.96	40.28 ± 1.53
Con A		52.49 ± 7.21	26.45 ± 6.76	48.32 ± 8.35	36.59 ± 9.76
PHA		13.23 ± 1.78	16.50 ± 3.14	19.44 ± 4.71	18.24 ± 4.78

[a]HCCM II and CM were separated into two fractions, <100,000 MW and >100,000 MW as indicated by <100 and >100, respectively. Sample fractions were supplemented to cultures at 200 µl/ml.

[b]Values represent cpm ± SEM (x 10^{-3}) for triplicate wells.

[c,d]Different superscripts designate between treatment differences in ^{3}H-thymidine incorporation ($p<.01$; Tukey mean comparisons). There was no treatment x rIL-2 interaction ($P>.05$).

[e]Values represent cpm ± SEM (x 10^{-3}) for quadruplicate wells.

*Designates significant difference as compared to other rIL-2 levels within column ($p<.05$; Tukey).

**Designates within row difference as compared to the similar MW fraction of CM ($p<.05$; Tukey).

TABLE 8. Fluorescence activated cell sorter analysis[a].

	Treatment[b]				
mAb	**Control**	**PHA**	**PHA+**	**PWM**	**PWM+**
HT23A (Total T)	670 ± 55	706 ± .5	640 ± 40	681 ± 75	662 ± 32
HT14A (T subset)	77 ± 11	162 ± 16	150 ± 28	111 ± 8	95 ± 23
IgG-FITC (B cells)	191 ± 59	429 ± 10	409 ± 17	427 ± 20	328 ± 8
Change in T cells ($x10^3$):		+ 36	- 30	+ 11	- 8
Change in B cells ($x10^3$):		+ 238	+ 218	+ 236	+ 137

[a]Values represent average number of antibody-labeled cells/ml ± SEM ($x\ 10^{-3}$) for two lymphocyte populations and the average change in cell number as compared to the control.

[b]Culture treatments are: Control - no mitogen or HCCM; PHA - PHA-stimulated; PHA+ - PHA-stimulated and HCCM II treated; PWM - PWM-stimulated; PWM+ - PWM-stimulated and HCCM II treated.

cells/ml) and an even greater decrease in B cells was also observed (-99,000 cells/ml). The presence of HCCM in PHA- and PWM-stimulated cultures decreased the T suppressor cell subset by 12,000 and 16,000 cells/ml, respectively, as compared to stimulated controls. All mitogen-stimulated cell populations contained a greater number of B cells than did the control cultures, but the T cell population decreased below control culture levels when HCCM was supplemented to the stimulated cultures (-30,000 and -8,000). The change in the approximate number of blast cells detected by side and forward light scatter analysis correlated with what was observed with final cell concentration in the FACS cultures and with ^{3}H-thymidine uptake data from the lymphocyte proliferation assays (Fig.14). Compared to PHA- and PWM-stimulated controls, the average percent blast cells in HCCM-treated cultures decreased from 42.8 to 25.8 and 42.2 to 8.9 %, respectively.

DISCUSSION

T lymphocytes are largely responsible for the actions of cell-mediated immunity. Allograft rejection is caused mainly by T cells, although antibodies produced by B cells can also play an important role. The early developing fetus and the protective placental tissue surrounding it, express antigens that are foreign to the mother (Chatterjee-Hasrouni and Lala, 1979; Jenkinson and Owen 1980; Billington and Burrows, 1986; Zuckermann and Head, 1986; Allen et al., 1987). During successful pregnancies, fetal antigens do not initiate a cell-mediated rejection response, even though immune cells are present at the feto-maternal interface (Beer et al., 1975). An immunosuppressive substance (HCCM-SF) produced by the conceptus and

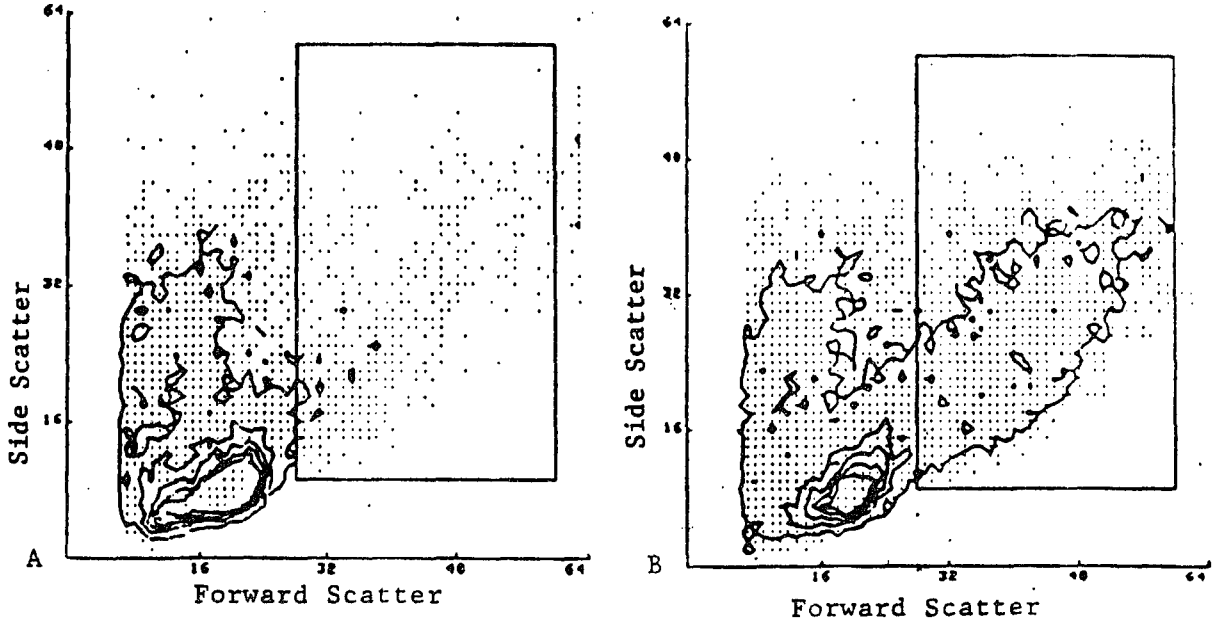

Figure 14. Two-dimensional scatter analysis with side and forward light scatter parameters was used to examine the effect of HCCM on cell blastogenesis. The blast cell populations in HCCM-treated and non-HCCM-treated, mitogen-stimulated cultures were compared. Gate settings were adjusted by comparing the mitogen-stimulated control to the non-stimulated control, such that the window contained the blast cells present only in the stimulated cell population. The same settings were then applied to the 2-D scatter plot of the HCCM-treated cell population.
A. PWM-stimulated, HCCM-treated cell population (PWM+) of sample #384. The same window used in A was used to determine the percent blast cells (5.2%).
B. PWM-stimulated cell population (PWM) of sample #384. Window contains the larger cells (blast cells; 33.3%) that were absent from the non-stimulated cell population.

acting on the T cells may be inhibiting the T cell response to fetal antigens, thereby interfering with the otherwise eminent allograft rejection response. Likewise, this localized T cell suppression could also affect B cell proliferation, resulting in a reduction in antibody production at the site of implantation. Maternal systemic immune response, away from localized HCCM-SF, would be unaffected. This hypothesis would explain why immune function of pregnant females is not compromised and anti-fetal antibodies are found in maternal circulation of 90% of normal equine pregnancies (Allen et al., 1987), yet the conceptus remains unharmed.

Through the use of PHA, a potent T cell stimulator, PWM, a T cell-dependent B cell stimulator and LPS, a T cell-independent B cell stimulator (Sharon, 1983; Jelinek and Lipsky, 1987), we obtained evidence indicating that the T cell population is directly affected by HCCM-SF. Possibly due to the potent stimulatory effect of PHA on T cells, relatively high concentrations (10-20%) of HCCM were required before a decrease in cell proliferation was observed. Supporting evidence for the suppression of T cells by HCCM was provided by the FACS analysis results which show the presence of HCCM decreased the T cell population by 66,000 cells/ml as compared to PHA-stimulated control cultures. While PHA-stimulation caused a dramatic increase in the B cell population compared to the control, there was only a minimal decrease of B cells when HCCM was also present in the culture. In contrast to PHA-stimulated cultures, PWM-stimulated cells appeared to be highly vulnerable to HCCM suppression as indicated by a rapid decline in their proliferative response at very low HCCM concentrations. The FACS data indicated that with PWM-stimulation

there was only a minimal difference between T cell populations of HCCM-treated and non-treated cultures, but a dramatic decrease in B cell number. Because B cell proliferation depends on T cell-derived lymphokines (Howard and Paul, 1983), HCCM suppression of T cells would inhibit the production of T cell factors, thereby indirectly affecting the B cell population. Since PWM is a weak T cell stimulator, the loss of even a small portion of T cells may decrease lymphokine production to such low concentrations that a severe reduction of B cell proliferation, as that observed with the FACS analysis, results. The hypothesis that HCCM does not act directly on B cells is further supported by the fact that HCCM is ineffective at suppressing proliferation of cultures stimulated with LPS. As LPS acts on B cells without a requirement for T cells, the effect of HCCM on the T cell population does not alter the B cell response to LPS. The overall effect of HCCM-SF on mitogen-stimulated blastogenesis is most visually obvious in the FACS two-dimensional scatter analysis. Since light scatter parameters allow the sorting of cells by size and density, the blast cells can be identified. The two-dimensional scatter plot quite effectively demonstrates the decrease in blast cells when HCCM is present in the culture.

The process from initial lymphocyte stimulation to actual cell proliferation involves critical intermediate events. Mitogens will initiate cell activation within the first 6 h of culture and lymphokine production follows (Weiss et al., 1987). Peak levels of lymphokines are produced 24 to 48 h after initial stimulation, but after 24 h, there is an increased expression of lymphokine receptors and the cells begin to use the lymphokines produced (Altman et al.,

1990). The lymphokine-receptor interaction acts as a secondary stimulus for promoting activated cell proliferation. Both B and T lymphocytes require T cell derived lymphokines such as IL-4 and IL-2, respectively. Therefore, if T cell activation or T cell production of lymphokines is inhibited, both B and T cell proliferation will be suppressed. Data from experiments relating to the temporal effect of HCCM on stimulated lymphocytes support the hypothesis that HCCM inhibits lymphokine production and/or lymphokine receptor expression by T cells. HCCM is largely ineffective at suppressing cell proliferation if added 48 h after the start of stimulation. Since most cells have received both primary (mitogen) and secondary (lymphokine) stimulation at 48 h, these results indicate that HCCM-SF must interfere with these initial events and that it does not affect the later events involved with proliferation. Treating cells with HCCM 24 h after the start of stimulation allowed the cells time to become activated by mitogen and to begin producing lymphokines, however, few of the cells had been fully stimulated by the secondary lymphokine-receptor interaction prior to HCCM treatment, and thus, the suppressor factor was effective.

Although little is known about how HCCM-SF acts on the cells, we have shown that pre-treatment of cells for 24 h with HCCM, followed by thorough rinsing, resulted in a cell population with full proliferative capacity when subsequently stimulated with mitogen. These data demonstrate many important characteristics of HCCM-SF. The factor is obviously not cytotoxic and exhibits only a transient effect on lymphocyte proliferation. Furthermore, the fact that HCCM treatment does not exhibit any long-term effects on a cell population's

responsiveness, indicates that HCCM suppression is not mediated through T suppressor cell recruitment and/or activation. The FACS data support this conclusion by demonstrating a decreased number of T suppressor cells in cultures treated with HCCM. Because proliferation of T lymphocytes depends largely on their ability to produce and respond to IL-2, an efficient mechanism for suppressing these cells would be an inhibition of IL-2 production. There is in vivo evidence that administration of antibodies to IL-2 can extend allograft acceptance (Kirkman et al., 1985). Furthermore, administration of a high IL-2 dose to mice terminates a successful ongoing pregnancy (Lala et al., 1990). It seems IL-2 plays an integral role in allograft acceptance and rejection. Considering this association, it is not surprising that many studies have been carried out examining the effect of conceptus or uterine derived suppressor factors on lymphocyte production of IL-2 and/or expression of IL-2R. In many cases the data indicate that a relationship exists between these suppressor factors and IL-2 production (Segerson, 1988; Saito et al., 1990; Segerson and Gunsett, 1990; Segerson and Libby, 1990). Since HCCM-SF acts on T lymphocytes and is only effective if present during the first 48 h of stimulation, it is possible the suppressor mechanism is mediated through the inhibition of IL-2 production. Our initial attempt to overcome a potential deficiency of IL-2 in HCCM-treated cultures by supplementing the cultures with PBMCS, was not particularly effective. Since it has been shown that stimulated equine lymphocyte supernatant contains the lymphokines required to support IL-2 dependent cell responses (Magnuson et al., 1984), we expected our PBMCS to contain high levels of IL-2, but our attempt to quantify this

IL-2 was unsuccessful because the murine-derived CTLL-2 cells did not respond to equine PBMCS.

Our attempt to override the suppressive activity of HCCM by supplementing cultures with rIL-2 at 200 U/ml also proved unsuccessful. The addition of this high concentration of rIL-2 should compensate for an IL-2 deficiency in the cultures. The fact that HCCM suppressed proliferation of cells, even in the presence of an over-abundance of rIL-2, demonstrates that HCCM suppression is not solely a result of deficient IL-2 production. Furthermore, because the slopes of the treatment curves in Figure 12 are the same, we can conclude that the suppressive activity of HCCM remains the same, regardless of the IL-2 concentration in the culture. Because a sub-optimal dose of PHA was used in the cultures, an IL-2 deficiency existed and the enhanced proliferation observed in the rIL-2 supplemented cultures simply reflects the effect of sufficient IL-2 on those cells not suppressed by HCCM-SF. One explanation for the inability of rIL-2 to overcome HCCM-induced suppression is that HCCM-SF is inhibiting IL-2R expression, rendering some cells incapable of responding to the exogenous rIL-2. Alternatively, HCCM-SF may be acting by interfering with the necessary IL-2/IL-2R interactions. Finally, as very high levels of IL-2 will activate T and B cells via the intermediate affinity p-75 ß receptor (Cosman et al., 1987), it is possible the high concentration of rIL-2 supplemented to the cultures stimulated cells not expressing the high affinity IL-2R, thereby increasing proliferation in the HCCM-treated cultures.

Because CTLL-2 cells continuously express the high affinity IL-2R, they were used to test the hypothesis that HCCM-SF interferes with

the IL-2/IL-2R interaction. We found that only the <100,000 MW fraction of HCCM inhibits CTLL-2 cell proliferation, possibly due to hormones and/or interferons produced by the horse conceptus. More important, our data show that the >100,000 MW fraction of HCCM exerts no suppressive effect on the CTLL-2 cell response to rIL-2. The fact that murine splenocytes are suppressed if treated with 200 µl/ml of the >100,000 MW fraction of HCCM (the same concentration used on the CTLL-2 cells), indicates that the ineffectiveness of HCCM on CTLL-2 cells is not simply due to species specificity of the suppressor factor. These results suggest that HCCM-SF interferes with cellular events prior to the expression of the high affinity IL-2R, but is not capable of suppressing cell proliferation following IL-2R expression. However, there does exist the possibility that the CTLL-2 cells differ significantly from T cells found in vivo, and thus may not be vulnerable to the suppressor activity of HCCM-SF.

Because HCCM-SF does not interfere with the IL-2/IL-2R interaction, we investigated the possibility that the high levels of exogenous rIL-2 activate T and B cells via the intermediate affinity p-75 ß receptor, thereby increasing proliferation in HCCM-treated cultures. In Figure 13 we show that with increasing rIL-2 concentrations, proliferation in non-stimulated cultures increased and then plateaued. The initial increase could be attributed to the effect of high rIL-2 levels on the p-75 ß receptors. A rIL-2 associated increase in proliferation was also observed in cultures of both PHA-stimulated treatment groups because sub-optimal PHA was used, and thus, IL-2 was deficient until cultures were supplemented with rIL-2. If rIL-2 supplementation increased proliferation of HCCM-treated

cultures only by activating cells via intermediate affinity receptors, a dose-dependent increase in proliferation would not result, and the slope of the PHA + HCCM treatment curve would be the same as that of the non-stimulated treatment curve. In contrast, the slope of the PHA + HCCM curve was the same as that of the PHA curve, indicating proliferation was mediated via high affinity IL-2R on activated cells. However, it is important to note that the relative difference in proliferation between these two treatments remained constant at all levels of rIL-2, indicating that, while the effect of rIL-2 was similar in both treatments, there were fewer cells capable of responding to the rIL-2 in the PHA + HCCM cultures. The results from Figures 12 and 13 and the CTLL-2 assay suggest that, in cultures treated with HCCM, there exist fewer IL-2R expressing cells capable of responding to rIL-2. These results are in agreement with reports by Bulmer and Johnson (1986) who found that T lymphocytes in human decidua do not express IL-2Rs. Still, there seems to exist some activated cells that do express the IL-2R and exogenous rIL-2 enhances their proliferative response. It is not known whether IL-2R expression is inhibited by HCCM-SF directly or indirectly through the interference of HCCM-SF with other early events in T cell activation. T cell expression of the high affinity IL-2R requires multiple signals, including antigenic stimulation in the context of self-major histocompatibility antigens and the macrophage product IL-1 (Gery et al., 1972). Our use of mitogens to stimulate the cells obviated the need for anitigenic stimulation, thus precluding the possibility that suppression involved an alteration in the function of the T cell antigen-specific receptor. We cannot rule out the possibility that

suppression results from an alteration in the production or function of IL-1. Such a mechanism of suppression has been suggested for a human placental protein (Pockley and Bolton, 1989).

If HCCM-SF is interfering with IL-2R expression, it is possible that it is actually acting directly on both B and T cells. PWM-stimulated B cells express the IL-2R (Waldmann et al., 1984) while LPS-stimulated B cells do not (Leibson et al., 1981; Robb et al., 1981), thereby indicating that IL-2Rs play an important role in PWM-stimulated B cell proliferation. HCCM-SF inhibition of IL-2R expression may imply a mechanism acting directly on B cells and indirectly, through T cell suppression, resulting in the loss of B cells and lack of ^{3}H-thymidine incorporation in PWM-stimulated, HCCM-treated cultures. Furthermore, as neither IL-2Rs nor T cells seem to be involved with LPS-stimulated B cell proliferation, it would follow that we would not observe suppression of such cultures with HCCM supplementation.

In a previous study, we reported the production of an immunosuppressive factor produced by Day 10 - Day 26 horse conceptuses (Roth et al., 1990). In that study, the conceptus cultures were carried out as those described herein for obtaining HCCM I. In this study, we cultured Day 20 conceptuses in medium without FCS, thereby obtaining HCCM II. The immunosuppressive characteristics of HCCM I and II appear to be similar, indicating that supplementation of FCS to the culture medium is not required by horse conceptus tissue for the production of the suppressor factor. HCCM-SF is >100,000 MW, suppresses proliferation of T lymphocytes and seems to indirectly suppress B lymphocytes. This non-cytotoxic suppressor factor is

effective only if present during the early stages of lymphocyte activation and its effect on cells is completely reversible. It is important that the suppressor factor is not cytotoxic, as excessive cell death at the feto-maternal interface could lead to the release of lysozymes and interferons resulting in an unhealthy environment for placental development. Furthermore, the lack of toxicity and the transient suppressive effect of HCCM, ensures that its effect is localized and that the systemic maternal immune system is not down-regulated or detrimentally altered by HCCM-SF during pregnancy. HCCM suppression is highly dependent on the suppressor factor's ability to act on the cells during the early stages of activation and could explain why such factors are produced by horse embryos as early as Day 10 (Roth et al., 1990). At this stage of development, the embryo is still highly mobile and is enclosed in the protective embryonic capsule. By day 15, when uterine migration ceases (Ginther, 1986) and the conceptus becomes more intimately associated with maternal tissue, the concentration of suppressor factor in utero may be high enough that local maternal T cell quiescence is maintained. Because of its suppressive effect on T cells, HCCM-SF could play a role in preventing lymphocyte destruction of endometrial cups prior to day 120 of pregnancy. Our results suggest that the factor suppresses cell proliferation by preventing IL-2R expression. In view of the fact that T cells are largely responsible for allograft rejection, and the IL-2/IL-2R interaction is a prerequisite for complete T cell activation, we hypothesize that this suppressor factor, by inhibiting IL-2R expression, disrupts the T cell-mediated allograft rejection that might otherwise terminate pregnancy.

SUMMARY AND CONCLUSIONS

One aspect of the fetal-maternal immunological relationship was studied. In our initial experiment, immunosuppressive activity derived from sheep, goat and sheep x goat hybrid trophoblast tissue obtained at day 20 of gestation was examined. Medium conditioned by all three types of trophoblast similarly suppressed both sheep and goat lymphocytes. The results from this study indicate that suppressor factors are secreted by hybrid trophoblast at day 20 of gestation and these factors do not differ from those of sheep and goat trophoblast tissue in their ability to suppress PWM-stimulated lymphocytes.

All subsequent studies, in which conceptus-derived immunosuppressor activity was studied in greater detail, were carried out with the horse conceptus. The equine model was chosen because it is unique in that hybrid and interspecific pregnancies are successful, yet the characteristic events involved in placental formation and subsequent function differ depending on the particular type of pregnancy. Prior to this study, horse conceptus immunosuppressor factors had not been described. Therefore, we first identified the presence of a non-antigen specific immunosuppressive factor produced by horse embryos from day 9 through at least day 26 of development. While we refer to this factor in the singular tense, it is quite possible there is more than one factor responsible for the immunosuppressive activity.

Initially we determined that the suppressor factor is >30,000 MW and extremely stabile under harsh treatments. Due to its size and stability, the factor does not appear to be a hormone or interferon.

HCCM-SF exhibits its suppressive effect in a similar manner on donkey and horse lymphocytes, yet is less effective on the less-related goat lymphocytes. These results differ from those of the preliminary sheep, goat, hybrid study where the suppressor activity in the conditioned medium from these species did not exhibit species-specificity. This difference may simply reflect the increased sensitivity of the equine assay relative to that of the sheep/goat assay.

In the third experiment, the effect of indomethacin on the production of the suppressor factor was examined. Horse trophoblast tissue from 21 day-old conceptuses was cultured for 24 h in the presence or absence of 10^{-4} M indomethacin. The concentration of PGE_2 and the suppressive activity of the conditioned mediums were determined. While PGE_2 was reduced by 91%, the suppressive activity of the two mediums did not differ. Thus, in contrast to results from a number of studies with other species which have indicated the involvement of PGE_2 in conceptus-derived suppressor activity, our data indicate that the horse trophoblast-derived immunosuppressor is not associated with prostaglandin production.

In our final series of experiments we examined the suppressive mechanism of HCCM-SF. Using microconcentrator centrifugation, we found that HCCM-SF is >100,000 MW. Results obtained from the FACS analysis in which we compared HCCM-SF-treated to non-treated lymphocyte cultures, indicated that HCCM-SF acts directly on T lymphocytes and indirectly on B lymphocytes. This conclusion was largely confirmed with results obtained from HCCM-SF-treated lymphocyte proliferation cultures in which mitogens with different

specificities were used. In addition, we determined that this factor is not cytotoxic, affects cells during the early stages of activation and does not permanently suppress the lymphocytes. These characteristics of HCCM-SF are important for the following reasons: 1) if cytotoxic, HCCM-SF would cause excessive cell death at the fetal-maternal interface, likely resulting in an unhealthy environment for fetal development, 2) if the effect of HCCM-SF was cytotoxic and/or irreversible, it might detrimentally alter maternal systemic immunity and, 3) the fact that HCCM-SF must affect cells prior to their complete activation could explain why HCCM-SF is produced by embryos prior to the cessation of uterine migration and the initiation of implantation. Furthermore, we found that a high concentration of human rIL-2 could not override the suppressive effect of HCCM-SF in lymphocyte cultures. However, IL-2R expressing CTLL-2 cells were not inhibited by HCCM-SF. These results indicate that HCCM-SF inhibits T cell expression of the IL-2R.

In summary, a high-molecular-weight (>100,000 MW) immunosuppressive factor is produced by early and late pre-implantation horse conceptuses. This factor is very stabile, is not cytotoxic and is not associated with PGE_2. It effects T lymphocytes early during cell activation by inhibiting the expression of the IL-2R and, thereby, may play a role in disrupting T cell-mediated allograft rejection that might otherwise terminate pregnancy. While no difference in sheep, goat and sheep x goat hybrid trophoblast tissue-derived suppressor activity on sheep and goat lymphocytes was identified, HCCM-SF did exhibit some degree of species-specificity. Together, these data indicate an important immunoregulatory role for

soluble trophoblast-derived factors that may facilitate fetal allograft survival and that may also be associated with the success or failure of interspecies pregnancies. With this newly acquired knowledge about the effect of HCCM-SF on horse lymphocytes, we can begin to examine similar factors and their effects on maternal lymphocytes during interspecific and hybrid pregnancies to more accurately assess the role of these factors in fetal allograft protection.

Future Work

While a large body of information pertaining to HCCM-SF has been accumulated, a great deal remains unknown and is worthy of further investigation. The objectives of future studies might be to determine both the physical nature and the physiological activity of this factor(s). Column chromatography might prove to be a viable method for determining the actual size of HCCM-SF. Numerous techniques, such as charcoal extraction, lipid extraction, glycosidase digestion and dithiothreitol reduction, could potentially be used to biochemically define HCCM-SF. Although we have identified some of the effects of HCCM on lymphocytes, its mechanism of suppression is not yet fully understood. As IL-1 plays a major role in lymphocyte activation, both the effect of HCCM on IL-1 production and the ability of exogenous IL-1 to overcome HCCM suppression should be investigated. Studies could also be carried out to examine the effect of HCCM on intracellular events during cell activation, such as Ca+ release. The results of such studies might lead to a more complete understanding of how lymphocyte quiescence is maintained in HCCM-treated cultures. It might also be possible to obtain more direct evidence of IL-2R

involvement in T lymphocyte suppression by using ^{125}I-rIL-2 and directly measuring IL-2R on HCCM-treated vs. non-treated cell populations.

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APPENDIX

In addition to the studies described in Chapters II - V, several experiments were carried out that were never published and/or fully completed. The information gained from these additional experiments, although sometimes inconclusive and/or preliminary, may be useful to those specifically interested in characterizing conceptus-derived immunosuppressive factors. Therefore, I have included in this appendix a brief summary describing the studies, the rationale behind them and the results.

While concluding that HCCM-SF is >100,000 MW and therefore not a steroid hormone, we questioned the possibility that hormones attached to larger molecules (carrier proteins) could remain in the retentate fraction during centricon centrifugation and thus, be active in the >100,000 MW fraction. RIAs were used to determine the concentration of progesterone and estrogen in HCCM, and to determine if the steroids were passing through the centricon membrane. A sample of HCCM was separated by centricon centrifugation and each fraction (>100,000 and <100,000 MW) and an aliquot of unfractionated HCCM were analyzed. Results from the RIAs indicated that the concentrations of progesterone and total estrogen in the unfractionated sample of HCCM are much lower (2 to 7 ng/ml and 1 to 10 ng/ml, respectively) than those required for suppressing lymphocytes in proliferation assays. Furthermore, the fact that 75 to 100% of the hormones were identified in the <100,000 MW aliquot of the fractionated HCCM clearly indicated that the hormones were passing through the centricon membrane. These results support our conclusion that the suppressive activity of HCCM is not due to steroid hormones.

In addition to the studies described in chapter III, two experiments designed to alter the immunosuppressive activity of HCCM were carried out. In the first experiment, the pH of HCCM (pH = 8.2) was altered by the addition of 1N HCl or NaOH to become acidic (pH = 2.0) or basic (pH = 12.0), respectively. The two samples were maintained at room temperature for 1 hour, neutralized (pH = 8.0), fractionated through 10,000 MW exclusion centricons and tested for suppressive activity. While similarly treated CM exhibited no suppressive effect, both acid and base treated samples of HCCM retained significant suppressive activity. However, it is possible the activity was reduced from that of fresh HCCM. In a second experiment HCCM was subjected to protease digestion. Aliquots of HCCM and CM (800 µl; w/o FCS) were combined with 40 mg of protease beads and were rocked on a tube rocker for 24 hours at room temperature. Following digestion, samples were fractionated with a 100,000 MW exclusion centricon and retentate volumes (>100,000 MW) were tested for suppressive activity. Protease-treated HCCM exhibited suppressive activity equal to that of non-treated HCCM.

The protein content in samples of HCCM lacking any exogenous protein source, was determined using a Bio Rad protein determination assay. Aliquots of HCCM from 20-day-old conceptuses (n=6; each cultured in 20 ml RPMI) were analyzed. The protein content ranged from .025 to .078 ng/ml with an average of .055 ng/ml.

Polyacrylamide gel electrophoresis was carried out with concentrated samples of HCCM (5X; n=6). Separating gels (both 7.5% and 10% acrylamide) were stained with coomassie blue. While numerous bands stained uniformly across all samples, there were no bands

distinguishing proteins >100,000 MW on the gels. Furthermore, in lanes where only the >100,000 MW fraction of HCCM was applied, there were no distinct bands. However, there was evidence of staining at the top of the gel indicating the possible existence of high-molecular-weight proteins that never entered the separating gel.

VITA

Terri Lynn Roth was born to Julie and Bernie Roth on February 20, 1964 in San Diego, California. She graduated as valedictorian of her class in 1982 from Foothill High School in Pleasanton, California. Following high school, she attended the University of California Davis where, in March 1986, she received her Bachelor of Science degree in Animal Science graduating with high honors. She remained at U.C. Davis following her acceptance into the Animal Science graduate program and received her Master of Science degree in June 1988. Terri then accepted a Board of Regents Fellowship at Louisiana State University where she has pursued a Doctor of Philosophy degree in Animal Science. Upon completion of this degree, Terri will accept a post-doctoral position at the National Zoo in Washington D.C. where she will use both her knowledge and training in an effort to enhance the propagation and survival of endangered species.

DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Terri Lynn Roth

Major Field: Animal Science

Title of Dissertation: Conceptus-derived immunosuppressive factors and their role in fetal allograft protection.

Approved:

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

Date of Examination:

June 26, 1991