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Decidual Inflammatory Events Associated with Early Embryo Loss.

By

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A thesis submitted to the Faculty of Graduate Studies and Research, McGill University, in partial fulfillment of the requirements of the degree of Master of Science.

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Abstract

The maternal immune system exerts a key role in determining the outcome of mammalian pregnancy. It has been proposed that the high rate of early embryo loss (20-30%) in DBA/2-mated CBA/J female mice may be the consequence of a maternal non-specific immune rejection response to the allogeneic conceptus. Early embryo resorption in mice has been associated with the activation of natural killer like cells, macrophages, and production of TH1 inflammatory cytokines. To investigate and detect the earliest events in the activation of decidual NK cells and macrophages in initiating early embryo loss, gene expression analysis of two NK cell molecular markers, IFNγ and perforin mRNA, and two macrophage specific cytokines, TNFα and iNOS mRNA, was performed. Since IFNγ is also produced by CD4+ T cells, cell labeling with an anti-NK antibody (DX5), cell sorting, and RT-PCR gene expression analysis were performed to confirm the source of IFNγ mRNA in the decidua and spleen of DBA/2-mated CBA/J female. This thesis presents evidence that confirms a role for NK cells and macrophages in implantation and early embryo loss. RT-PCR analysis showed that IFNγ, perforin, TNFα, and iNOS mRNA positive cells selectively infiltrate all placentae as early as 24 hours following implantation (day 6 of gestation) and may be natural components of the implantation response. Second, increased expression of NK cell and macrophage molecular markers at day 8 of gestation, well before embryo damage was apparent, confirmed that infiltration of NK cells and macrophages was not a consequence of resorption. Third, RT-PCR at day 8 of gestation strongly suggested an association between increased expression of TNFα, iNOS, and IFNγ mRNA and resorption. RT-PCR further confirmed that perforin-mediated NK cytotoxicity was not directly responsible for embryo resorption, as perforin mRNA expression displayed a normal distribution among all the individual embryo implantation sites. Finally, expression of IFNγ mRNA by decidual DX5+ cells but not by DX5- cells demonstrated that NK cells could be the cellular source of decidual IFNγ mRNA. This study supports the premise that decidual NK cell production of IFNγ leads to macrophage activation and embryo demise. The role of the innate response during implantation, normal pregnancy, and early pregnancy loss will be discussed.

Résumé

Chez les mammifères, l'issue d'une grossesse dépend en grande partie du système immunitaire maternel. Il a été avancé que le taux élevé d'avortement prématuré qui touche les embryons des femelles CBA/J fécondées par les mâles DBA/2 pourrait être la conséquence d'un rejet immunitaire maternel non spécifique dirigé contre l'embryon allogénique. La résorption embryonnaire hâtive chez les souris a été associée avec l'activation des cellules NK et des macrophages de même qu'avec la production de cytokines inflammatoires de type Th1. Afin d'étudier les premières étapes de l'activation des cellules NK et des macrophages de la caduque basale induisant l'avortement spontané, une analyse d'expression génique a été effectuée. Cette analyse utilisa 2 marqueurs des cellules NK, l'ARN messager (ARNm) de l'interféron gamma (INF-γ) et l'ARNm de la perforine, de même que deux cytokines spécifiques aux macrophages, l'ARNm du facteur de nécrose tumorale alpha (FNT-a) et l'ARNm de l'oxyde d'azote (iNOS). Comme INF-γ est aussi produit par les cellules T CD4+, un traitement avec un anticorps dirigé contre les cellules NK (DX5) suivi d'un tri des cellules portant l'anticorps et une analyse d'expression génique (utilisant le RT-PCR) nous ont permis de confirmer la source de l'ARNm d'INF-γ retrouvé dans la caduque basale et dans la rate des femelles CBA/J fécondées par les mâles DBA/2. Cette thèse démontre des faits qui viennent confirmer le rôle des cellules NK et des macrophages au niveau de l'implantation et de la perte prématurée d'embryons. L'analyse du RT-PCR (reverse transcriptase-polymerase chain reaction) démontra que les cellules exprimant de l'ARNm d'INF-γ, de perforine, de FNT-a et de iNOS infiltrent de manière sélective tous les placentas moins de 24 heures après l'implantation (jour 6 de la période de gestation) et pourraient faire partie du cycle normal d'implantation. Deuxièmement, une hausse de l'expression des marqueurs moléculaires des cellules NK et des macrophages au 8e jour de gestation confirma que l'infiltration des cellules NK et des macrophages n'est pas une conséquence de la résorption car à ce moment, aucun dommage embryonnaire n'est apparent. Troisièmement, le RT-PCR du 8e jour de gestation suggéra une association entre l'expression à la hausse de l'ARNm du FNT-a, de l'iNOS et de l'INF-g et la résorption. Le RT- PCR confirmera aussi que la cytotoxicité des cellules NK due à la synthèse de

perforine n'était pas directement responsable de la résorption embryonnaire car l'expression de l'ARNm de la perforine démontre un distribution normale parmi tous les sites individuels d'implantation embryonnaire. Finalement l'expression de l'ARNm de l'INF-γ par les cellules de la caduque basale qui étaient DX5+ et non par celles qui étaient DX5- démontre pour la première fois que les cellules NK pourraient être la source de l'ARNm de l'INF-γ exprimé dans la caduque basale. Cette étude supporte l'idée voulant que la production d'INF-γ par les cellules NK de la caduque basale mène à l'activation des macrophages puis à la mort de l'embryon. Le rôle de la réponse innée durant la phase d'implantation, le développement normal d'une grossesse et la perte prématurée d'embryons seront les thèmes discutés dans cet ouvrage.

I dedicate this thesis to my parents, Antigone and Athanasios Merkouris, my sisters, Dimitra and Nektaria, and last but not least my loving husband Steve Magafas. I would not have been able to achieve this goal without your love, encouragement and support.

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Literature Review

1. Introduction

The immunological paradox posed by fetal survival during pregnancy has long intrigued immunologists (Baines and Gendron, 1993). In mammalian reproduction, the mating of histoincompatible individuals produces paternal antigen expressing concepti that are in direct contact with maternal uterine and blood-borne immune effectors (Billingham, 1964). Considering the immune system has the capacity to discriminate between self and non-self and reject foreign tissue grafts and pathogens, it would be rational to anticipate rejection of the "fetal allograft" by the maternal host (Raghupathy, 1997). However, in successful pregnancy the allogeneic conceptus is not rejected and its survival within the potentially hostile maternal environment is dependent on a poorly understood network of mechanisms in which the immune system itself appears to exert a key role.

In spite of the enormous growth in the human population, mammalian reproduction is not as efficient as it is commonly thought. In fact, between 40% to 60% of all implanted embryos are lost before term and an estimated two-thirds of these losses may be due to early embryo demise (Baines *et al.*, 1997; Baines Gendron, 1993). In humans, approximately 25% of normal embryos undergo spontaneous early abortion before the third week of gestation following the last menstrual period (Edmonds *et al.*, 1982; Lippman and Farookhi, 1986; Wilcox *et al.*, 1988). Since recent data from human and murine studies point to a putative immunological etiology for this type of pregnancy

failure (Carp *et al.*, 1990; Chaouat *et al.*, 1988), this thesis will focus on the immunological factors associated with implantation and early embryo loss.

2. The Maternal-Trophoblast Interface

Vital to understanding the immune events during pregnancy and early embryo loss, is the close relationship between trophoblast cells and maternal decidua at the fetomaternal interface. Embryonic development and establishment of the fetomaternal interface is a multistep process that may be divided into two stages: the preimplantation stage and the post-implantation stage. In the preimplantation stage, the fertilized ovum undergoes a series of cleavage and differentiation events to form the blastocyst. Trophoblast cells that form the outer layer of the blastocyst then implant into the uterine wall, induce endometrial decidualization, and establish direct contact with maternal blood sinusoids and decidual tissue (Enders, 1991; Arck and Clark, 1997). The inner cell mass of the blastocyst eventually differentiates into the fetus while the trophoblast becomes the placenta. Since the trophoblasts line all areas of contact between the fetus and the mother, the trophoblast cells rather than the fetus *per se* must resist immune rejection by blood-borne and decidua-associated immune effectors (Torry *et al.*, 1997). In humans and mice, the vascular deciduum contains enlarged glycogen-filled stromal cells, lymphomyeloid cells and various populations of immune effector cells that are thought to play pivotal roles in determining the outcome of pregnancy. These include: macrophages, natural killer cells (NK), granulocytes, γδ T lymphocytes and a distinct population of granulated uterine natural killer lineage cells that are found in the

mesometrial triangle in early murine and human pregnancy. A review of decidua-associated effectors in early embryo loss will be presented later in this chapter.

3. Reproductive Failure

A brief discussion of the modes of spontaneous abortion and an animal model facilitating its study will be presented in order to put early embryo loss into perspective.

(a) Spontaneous Abortion

Although reproductive failure can take place at any time during gestation, the most common complication of mammalian pregnancy is spontaneous abortion (Tangri and Raghupathy, 1993; Clark and Chaouat, 1989; Hill *et al.*, 1995). It has been estimated that as much as 60% (Edmonds *et al.*, 1982; Wilcox *et al.*, 1988; Baines and Gendron, 1990) of all human implanted pregnancies are spontaneously aborted within the first 14 weeks of gestation (Clark and Chaouat, 1989) and despite many years of intense research, the underlying causes in many cases remain unknown (Raghupathy, 1997; Arck and Clark, 1997). Since clinical studies involving couples with a history of recurrent spontaneous abortion (RSA) have shown that many early abortions are not explained by conventional endocrinological and anatomical abnormalities (Hill and Ravinikar, 1990), exogenous pathogens (Benirschke and Robb, 1987), and chromosomal or genetic defects (Stray and Stray-Pedersen, 1984), recent attention has focused on immunological etiologies.

Supporting the contention of an immunological etiology in some cases of spontaneous abortion, is the benefit observed when women who habitually abort their

babies undergo active immunotherapy. For instance, studies by Beer et al (1981), Mowbray et al (1985) and Carp et al (1990) showed that alloimmunization of women who habitually abort their concepti with paternal lymphocytes or pooled allogeneic lymphocytes, improved reproductive performance to a significant extent. Likewise, a meta-analysis of the controversial intravenous immunoglobulin (IVIG) therapy for spontaneous abortion, showed that in some cases, IVIG treatment did improve the outcome of pregnancy (Daya *et al.*, 1998).

On the other hand, recent analyses, by Coulam and colleagues in 1996, of clinical trials, argue against the benefit of immunotherapy for RSA. These studies show that allogeneic leukocyte immunization and IVIG had little or no effects in RSA and that chromosomal abnormalities were a significant confounder to treatment success. The apparent contradictory effects of immunotherapy may in part be explained by new data that links up to half of the cases of recurrent spontaneous abortion with karyotypic abnormalities in the trophoblast (Clark and Coulam, 1996; Stern *et al.*, 1996). It has been suggested that perhaps only karyotypically normal pregnancies could benfit from immunotherapy.

In humans, approximately 25% of implanted embryos spontaneously cease development within 7 to 14 days following attachment to the endometrium (Edmonds *et al.*, 1982; Lippman and Farookhi, 1986; Wilcox *et al.*, 1988). The pathophysiology of early embryo loss may differ from spontaneous fetal abortions. The latter usually occurs after the sixth week of gestation, and often results from problems of maternal physiology or fetal development, and is detected in increased frequency in patients with recurrent spontaneous abortion (Hafez, 1984; Clark and Chaouat, 1989). Conversely, spontaneous

early embryo resorption may be associated with developmental failure or embryo destruction by immunological mechanisms. Embryo loss usually takes place without the expulsion of any visible products of conception, and is seldom detected by the mother (Baines and Gendron, 1993). Furthermore, some researchers have speculated that this mode of pregnancy loss may have a higher incidence in some couples with primary infertility. In this thesis, the focus will be on spontaneous early embryo loss.

(b) Difficulties Associated with Human Study of Early Embryo Loss

Various obstacles are associated with human study of early embryo loss. First, the low and unpredictable incidence of early embryo wastage in documented human pregnancies causes scarcity of relevant tissue samples and products of conception that are needed for complete cellular and molecular analysis of the decidua-trophoblast interface. Second, from an ethical and practical point of view, it is still impossible to predict early embryo demise in couples with a history of primary infertility or recurrent spontaneous abortion. Finally, many clinical studies have been unreliable and contradicted by data obtained from prospective and experimental studies (Baines and Gendron, 1993).

(c). Early Embryo Loss in a Murine Mating Model

The murine mating model, CBA/J (H2-k) females X DBA/2 (H2-d) males, has greatly facilitated the investigation of the cellular and molecular factors present at the decidua:trophoblast interface during early embryo loss. In this abortion-prone combination, 20-30% of implanted embryos are spontaneously resorbed by day 12 of gestation, similar to the frequency of early embryo loss seen in humans (Clark *et al.*,

1980; Clark and Chaouat, 1989). The losses are recurrent, increase with the aging female, and are partner specific since other H-2k X H-2d murine mating combinations demonstrate significantly lower early embryo loss rates of 5-10% (Chaouat *et al.*, 1988; Chaouat and Menu, 1997; Duclos *et al.*, 1995). Furthermore, histological examination has demonstrated that resorption is characterized by focal necrosis at the junction of trophoblast with decidua, by a large infiltration of polymorphonuclear leukocytes, and by thrombosis and hemorrhage (Duclos *et al.*, 1995; Clark *et al.*, 1998).

Since the mating of inbred mice significantly decreases the occurrence of lethal genetic anomalies in the resulting embryos, and only 4-7% of spontaneous abortions in mice have been documented to result from chromosomal abnormalities (Stern *et al.*, 1996; Arck *et al.*, 1999), researchers have focused on findings that suggest an immunological basis for the increased rate of embryo demise in DBA/2-mated CBA/J mice. The most striking evidence supporting an immunological role, is the effect of alloimmunization in decreasing the resorption rate to normal levels. For instance, immunization of CBA/J females with BALB/C (H-2d) lymphoid cells before mating with DBA/2 males restored normal embryo viability (Kiger *et al.*, 1985). Likewise, adoptive transfer of T cells from an immunized CBA/J female to virgin CBA/J females before mating with DBA/2 males similarly reduced the resorption rate (Chaouat *et al.*, 1988; Tangri and Raghupathy, 1993). Furthermore, non-specific immunotherapy with Complete Freund's Adjuvant (CFA) has been shown to enhance embryo survival via progesterone mediated immunomodulation (Skekeres-Bartho *et al.*, 1991). Given that the effects of treatment on embryo survival were not permanent as would be expected from specific anti-paternal enhancing antibodies and suppressor cells, it has been suggested

that the effects are most likely due to non-specifc immunomodulation (Baines *et al.*, 1996). Interestingly, immunization was accompanied by an increased production and activity of non-specific immunomodulatory factors that have been associated with successful pregnancy, such as decidual IL-10, and TGF-beta2 producing suppressor cells (Chaouat *et al.*, 1995; Arck and Clark, 1997).

Although the exact immunological mechanisms that initiate and mediate murine early embryo loss have not yet been fully elucidated, it appears that embryo demise may result from impaired placental development. As will be discussed in later sections, this placental damage is thought to be dependent upon the activation of the non-specific components of the maternal immune system (natural killer cells and macrophages) rather than CD4+ or CD8+ T lymphocytes and antibodies that characterize specific immune responses (Gendron *et al.*, 1990; Duclos *et al.*, 1995; Haddad *et al.*, 1997a). Furthermore, studies involving the modulation of pregnancy outcome with abortifacient factors or resorption inhibitors, also attribute early embryo wastage to TH1 cytokines (Raghupathy, 1997) and to cytokine-triggered thrombotic/inflammatory processes in maternal uteroplacental blood vessels (Clark *et al.*, 1998).

4. Current Theories Explaining Fetal Survival and Early Pregnancy Loss

The ability of the semi-allogeneic fetus to survive in its immunocompetent host has lead researchers to wonder how the maternal rejection response is regulated during gestation, and to what extent is early pregnancy loss associated with aberrant immunoregulation. A number of theories have been proposed and combine to explain the immunological components involved in fetal acceptance and early pregnancy failure. In

this section, the theories are divided into general non-specific mechanisms and antigen-specific mechanisms.

(a) Non-Specific Mechanisms

(i) Immunosuppression

Initially, it had been postulated that the fetus evades immune rejection by inducing an immunosuppressive state in its maternal host. Supporting this view, were studies revealing a gestationally-induced decline of certain components of local and systemic cell-mediated immunity, namely delayed type hypersensitivity (DTH) and natural killer cell responses (Luft and Remington, 1984; Holland *et al.*, 1984), that predominated during conventional tissue allograft rejection. These studies linked pregnancy with reactivated cytomegalovirus infections (Mclaughlin, 1990), susceptibility to several types of intracellular infectious diseases including tuberculosis (Jameson, 1935), toxoplasmosis (Luft and Remington., 1982), and malaria (Watkinson and Rushton, 1983), and improvement of cell mediated autoimmune diseases such as rheumatoid arthritis (DaSilva and Spector, 1992). Another study indicating a gestationally-induced change in immune competency, involved the local infection of murine decidua with *Listeria monocytogenes* (Redline and Lu, 1987). In this experimental system, pregnant mice could not mount adequate cell mediated responses that were needed for pathogen rejection and clearance. Finally, an increased frequency of resorption in C57B16 mice resistant to local decidual infection of *Leishmania major* demonstrated that cell mediated responses and TH1 cytokines could have deleterious effect on fetal survival (Krishnan *et al.*, 1996).

Given that most pregnant women were not exceedingly vulnerable to infections (Sacks, 1999), and antibody mediated immunity was relatively normal during gestation (Wegmann *et al.*, 1993), it stood to reason, then, that pregnancy was not a state of total immunosuppression. Current explanation of the immunosuppressive theory is based on a large body of evidence indicating that successful pregnancy is associated with increased local production of maternally and fetally-derived molecules and cells that have the capacity to suppress local maternal cell-mediated immune rejection responses (Clark *et al.*, 1997). Moreover, a reduction of these factors or the cells that produce them may lead to placental failure and early embryo demise. Examples of such factors are transforming growth factor beta (TGFβ), IL-10 and the hormone progesterone.

For the last two decades, a great deal of work has gone into characterizing and identifying the cells that produce local suppressor factors. Recently, analyses of decidua from DBA/2-mated CBA/J female mice have identified a population of small granulated lymphocytes that secrete *in situ* a suppressor effect resembling TGFβ2 (Lea *et al.*, 1992; Arck and Clark, 1997). This TGFβ2 suppressive factor is antigen nonspecific and has the capacity to inhibit the activation and generation of potentially abortogenic effectors at the fetomaternal interface, namely natural killer cells, macrophages, and lymphokine activated killer cells (LAK) (Arck and Clark, 1997). Characterization of these decidual natural suppressor cells revealed that they lack T cell markers such as Thy1, Lyt1, CD4 and CD8, and the natural killer cell marker asialoGM1, but may be of T cell lineage because their suppressive activity could be abrogated by anti-CD3 and anti-TCR γδ treatment (Clark *et al.*, 1997). Additionally, suppressive activity from these cells was dependent on the presence of placental trophoblast. In light of evidence that γδ T cells

may recognize and react to trophoblast cells (Heyborne *et al.*, 1994), and γδ TCR+ lymphocytes from pregnant mouse uterus may produce TGFβ2 (Suzuki *et al.*, 1995; Clark *et al.*, 1997), it has been suggested that trophoblast may be stimulating γδ T cell suppressive activity in the CBA X DBA combination (Clark *et al.*, 1997). However, this is a description of only one type of murine natural suppressor cell. Definitive characterization of other suppressor cells and local TGFβ2 producing suppressor cells in humans and in other murine strains, such as T-cell deficient SCID mice (Clark *et al.*, 1994), remains elusive and a subject of on going debate and intense research.

IL-10 is another immunosupressive cytokine that has been detected in murine and human decidual and placental tissue (Chaouat *et al.*, 1995; Roth *et al.*, 1996). This TH2/3 immune mediator which is a potent down-regulator of inflammatory cytokines has been associated with successful human pregnancy (Hill *et al.*, 1995) and appears to play a positive role in preventing early embryo loss in DBA/2-mated CBA/J female mice. Studies involving the modulation of IL10 levels have shown that injection of the abortion-prone females with IL-10 prevents embryo demise. Alternatively, injection with anti IL-10 antibodies increases the resorption rate (Chaouat *et al.*, 1995).

The gestational hormone progesterone also plays an imunosuppressive role. During pregnancy, CD8+ T cells are activated to express progesterone receptors. In response to hormone, these T cells secrete progesterone induced blocking factor (PIBF), which acts by inhibiting natural killer cell cytolytic activity (Szekeres-Bartho *et al.*, 1989; Clark *et al.*, 1996). It has been demonstrated that PIBF could abrogate NK cell mediated resorption in mice, thus acting in favor of pregnancy maintenance (Szekeres-Bartho *et al.*, 1990). Furthermore, the addition of PIBF to cultured murine lymphocytes resulted in

increased production of TH2 inflammatory down-regulators such as IL-10 (Szerkeres-Bartho, 1996). Finally, progesterone has recently been shown to inhibit the *in vitro* production of tumor necrosis factor alpha (TNFα) by activated murine macrophages (Miller and Hunt, 1998), which have been associated with early embryo loss.

Evidence has accumulated supporting the contention that a reduction of locally derived immunosuppressive factors may lead to early pregnancy failure. For instance, resorption prone placentae from DBA/2-mated CBA/J mice display reduced levels of IL-10 compared with low loss mating combinations (Chaouat *et al.*, 1995). Human studies have revealed that a subset of human patients with a history of recurrent spontaneous abortion demonstrate a deficiency of TGFβ2-producing suppressor cells in uterine tissue near the placental attachment site (Lea *et al.*, 1995). Women with recurrent spontaneous unexplained abortion tend to have below normal levels of T cells expressing cell surface progesterone receptors (Clark and Coulam, 1996; Szekeres-Bartho *et al.*, 1989; Clark *et al.*, 1996). Finally, protection against resorption following alloimmunization in humans and mice is also accompanied by increased decidual production of TGFβ2 and IL-10 (Gafter *et al.*, 1997; Chaouat *et al.*, 1995).

(ii) Immunotrophism and Immunodystrophism

Another non-specific hypothesis attempting to explain immune regulation during pregnancy is immunotrophism. This mechanism was first proposed by Wegmann in 1986 (Tangri *et al.*, 1994; Raghupathy and Tangri, 1996; Raghupathy, 1997), and is based on the concept that maternal alloreactivity may actually promote fetal survival. The striking observation that vaccination (of CBA/J females prior to mating DBA/2

males) with paternal leukocytes not only enhanced embryo viability but increased placental weight, led to the proposal that anti-trophoblast responses may stimulate local secretion of trophic factors and cytokines that promote optimal growth and functioning of the placenta. Colony stimulating factor 1 (CSF-1), IL-3, and granulocyte monocyte-colony stimulating factor (GM-CSF) are examples of trophic cytokines that have been shown to promote the growth of mouse and human trophoblast (Athanassakis *et al.*, 1987; Armstrong and Chaouat, 1989; Wegmann *et al.*, 1993). Additionally, maternal T lymphocytes have been implicated as producers of these factors, as anti-CD4 or anti-CD8 antibody treatments were shown to diminish placental cell proliferation, reduce fetal size, and increase the early embryo loss rate in resorption prone mating pairs (Athanassakis *et al.*, 1990). However, the notion of T cells as the sole sources of immunotrophic factors is weakened by various lines of evidence. First, CSF-1 is not produced by T cells but by macrophages, fibroblasts and endothelial cells (Hill, 1992). Second, normal resorption rates and fetal weights seen in GM-CSF knockout female mice when mated by wild type males has been attributed to embryonic production of GM-CSF (Robertson *et al.*, 1999). Third, successful pregnancy is observed in SCID mice even though they are T cell deficient.

Since trophic factors are thought to be beneficial to pregnancy, researchers have reasoned that a reduction or deficiency in these cytokines may lead to placental failure and early embryo demise (Clark and Chaouat, 1989; Raghupathy and Tangri, 1996). Indeed, moderate fertility impairment has been seen in GM-CSF knockout mice (Robertson *et al.*, 1999) and administration of small amounts of this cytokine may correct the increased loss rates in the CBA/J X DBA/2 resorption prone combination.

Immunodystrophic effects may also be the cause reproductive failure. According to this hypothesis, maternal anti-trophoblast responses may induce the overproduction of detrimental cytokines that can result in negative consequences for the developing conceptus (Hill, 1991; Raghupathy and Tangri, 1996). Examples of dystrophic cytokines are tumor necrosis factor alpha (TNFα) and interferon gamma (IFNγ). As will be seen later in this thesis, these inflammatory cytokines have been associated with the mechanisms preceding early embryo loss in DBA/2-mated CBA/J female mice (Haddad *et al.*, 1997a;b).

(iii) Fas-FasL Interaction at the Fetomaternal Interface

Fas-Fas ligand (FasL) interaction is thought to play a protective role in the maintenance of immune privileged sites. Immune privileged sites are certain areas of the body that share a unique relationship with the immune response. They tend to be tissue sites which are more permissive to allografts, are unable to tolerate even short episodes of inflammation, and actively prohibit the occurrence of inflammatory responses (Griffith and Ferguson, 1997). As with the eye, the murine testis and hamster cheek pouch, the uteroplacental unit during pregnancy has sometimes been referred to as an immune privileged site. Recent identification of FasL expression at the maternal-fetal interface in both mice (Hunt *et al.*, 1997c; Uckan *et al.*, 1997) and humans (Hammer *et al.*, 1999), has led to the proposal that FasL induced apoptosis or "programmed cell death" of effector lymphocytes may protect the allogeneic conceptus against Fas+ immune cell attack (Hunt *et al.*, 1997c).

FasL or CD95 ligand is a type II membrane protein and a member of the tumor necrosis factor family. Also called "death factor", it is expressed by activated cytotoxic T lymphocytes, TH1 cells, natural killer cells and by some nonlymphoid cells such as placental trophoblast. Upon crosslinking of FasL to the Fas "death" receptor (CD95), a death or apoptotic signal is transmitted to the lymphoid or non-lymphoid Fas-bearing target cell. FasL-Fas interaction controls the expansion of T cells during immune responses (Nagata and Golstein, 1995), maintains peripheral tolerance by clonally deleting self reactive T cells that may have escaped negative selection in the thymus, and is a killing mechanism used by cytotoxic T lymphocytes (Rouvier 1993; Griffith and Ferguson, 1997). Furthermore, Jiang and Vacchio (1998) have suggested that FasL on trophoblast may be one of the mechanisms mediating clonal deletion of fetal antigen reactive peripheral T cells during murine pregnancy.

By employing molecular technologies, Hunt and colleagues (1997c) have demonstrated that during murine pregnancy, FasL is appropriately positioned both in the uterus and in the placenta to protect the allogeneic conceptus from activated Fas-expressing effector cells of maternal origin. Furthermore, examination of uteroplacental sections from *gld* "general lymphoproliferative disease" homozygous matings, which express nonfunctional FasL, revealed that in the absence of FasL, maternal leukocytes flood the maternal-fetal interface and fetal survival is reduced. Considering that human natural killer like cells and T cells have been shown to express Fas upon activation (Eischen *et al.*, 1996; Saito *et al.*, 1994) and macrophages express Fas constitutionally (Liles *et al.*, 1996), it is possible that Fas expressing decidual leucocytes function as targets for FasL expressing trophoblast, thus maintaining the immune privileged nature

of the fetomaternal interface (Hammer *et al.*, 1999). However, the fact that successful pregnancy does occur both in *gld* mice and in Fas deficient *lpr* "lymphoproliferative" mice, makes further investigation into the relevancy of the FasL/Fas system at the maternal-fetal interface a necessity.

(b) Specific Mechanisms

(i) Placental Trophoblast Immunogenicity and MHC

Considerable attention has also been placed on the postulate that allogeneic concepti avoid immune rejection by evading the afferent arm of the immune system (Torry *et al.*, 1997). When an individual is challenged with foreign or "non-self" antigens, maternal immune detection or recognition (afferent arm) may subsequently lead to a rejection response. Invading antigens can be processed by innate antigen presenting cells (APC), and then presented to T lymphocytes in association with "self" polymorphic MHC (major histocompatibility complex) class I or II molecules. At this point, if appropriate costimulatory molecules and cytokines are present, specific cellular and humoral mediated immunity might become activated, thus eliminating the foreign entity (Sacks *et al.*, 1999). The nature of the ensuing response depends on whether antigen is presented on cells expressing MHC class I or II. While ubiquitously expressed MHC class I molecules present antigen to cytotoxic CD8 positive T lymphocytes, MHC class II molecules are expressed by immune cells and are recognized by CD4 positive T helper cells. T helper cells have been implicated in inflammatory processes as well as in activation of macrophages and antibody producing B-lymphocytes.

T cells also have the ability to recognize and bind to "non-self" MHC molecules expressed on transplanted tissue grafts, resulting in graft rejection (Yokoyama, 1997). It has been suggested that lack of T cell mediated rejection of the fetal allograft during successful pregnancy may in part be due to reduced or weakened trophoblast immunogenicity (Gill *et al.*, 1993; Saji, 1993). Supporting this theory is ample evidence indicating an altered or decreased expression of conventional MHC class I and class II antigens by the placental trophoblast cells that enclose the developing conceptus and come into direct contact with the maternal circulation. Indeed, studies of human placental tissues have indicated the total absence of polymorphic MHC class I and II molecules on trophoblast cells ((Hunt *et al.*, 1988; Faulk and Hunt, 1990). Murine placental trophoblast are also devoid of class II antigens and studies modulating their expression have supported the contention that absence of these polymorphic molecules on placental trophoblasts allows the allogeneic conceptus to escape immune detection and subsequent rejection. In particular, when mice were treated with drugs that induced MHC class II expression, fetal survival was compromised. When the same mice were then administered anti-class II antibodies, pregnancy loss was prevented (Athanassakis and Papamettheakis, 1991; Chaouat, 1993).

However, other findings have cast doubt upon the postulate that successful pregnancy requires evasion of immune recognition. For instance, specific immune mediators such as maternal anti-paternal antibodies (Jalali *et al.*, 1996; Mowbray *et al.*, 1997) and peripheral alloantigen reactive T cells (Jiang and Vacchio, 1998) are usually generated during normal gestation. Second, murine placental trophoblast cells that directly encounter the maternal circulation have been shown to express polymorphic

MHC class I molecules and it has been suggested that these molecules may actually play a protective role during pregnancy. MHC might bind and eliminate some of the alloantibodies generated against paternal placental antigens (Wegman, 1987; Chaouat 1993). Furthermore, human trophoblasts have been shown to express a monomorphic MHC class I molecule, human leukocyte antigen G (HLA-G), that is thought to protect against natural killer cell-mediated cytolysis by binding to NK cell killer inhibitory receptors (KIRs) (King *et al.*, 1997; Arck *et al.*, 1999; Rouas-Freiss *et al.*, 1997). Finally, immunostimulation of pregnant female mice with cells transfected to express allogeneic MHC antigens, has been shown to enhance embryo viability and correct resoption rates in abortion prone mating models (Menu *et al.*, 1995).

(ii) Protective Antibodies

Despite the association of pregnancy with enhanced adaptive humoral immunity, antibody-dependent cellular cytotoxicity (ADCC) effectors are not eminently apparent at the fetomaternal interface (Arck and Clark, 1997). Recently, it has been postulated that some of the maternal antibodies generated during gestation may actually protect the developing conceptus from immune destruction. The formation of blocking antibodies, antibody:antigen complexes, and anti-idiotypic antibodies has been proposed to specifically enhance fetal survival.

In humans, the existence of blocking antibodies and antigen:antibody complexes has been substantiated by the observation that in all successful term pregnancies, IgG antibodies are found bound to placental trophoblasts (Jalali *et al.*, 1989; Mowbray *et al.*, 1993). Further validation of this type of antibody mediated protection mechanism has

come from the identification of a novel 80-kDa allotypic trophoblast protein (R80K) to which IgG antibodies are made. R80K is polymorphic and remains completely covered with maternal IgG during pregnancy (Arck *et al.*, 1999). Experiments investigating the *in vitro* and *in vivo* effects of polyclonal anti-R80K antibodies (that have been eluted from human placenta) as well as murine monoclonal anti-R80K antibodies, have suggested that R80K is covered with blocking IgG during successful pregnancy in order to prevent natural killer cell mediated attack of trophoblasts (Jalali *et al.*, 1996). In one experiment, the addition of polyclonal anti-R80K antibodies to an assay, containing human natural killer and target cells inhibited NK mediated cytolysis. In another study, treatment of resorption prone DBA/2-mated CBA/J female mice (whose elevated resorption rate is thought to be dependent on NK cells) with murine monoclonal anti-R80K antibodies, significantly prevented early embryo demise. Although these studies did not address why IgG blocking antibodies do not initiate antibody dependent cellular cytotoxicity (ADCC) and cytopathology, it has been suggested that a deficiency in blocking antibodies may compromise the outcome pregnancy (Mowbray *et al.*, 1997).

In addition to blocking antibodies, several investigators have alluded to a potential regulatory role for trophoblast anti-idiotypic antibodies during successful pregnancy. An idiotype may be described as a collection of determinants within the antigen binding site of specific immunoglobulin molecules and lymphocyte antigen receptors, that are capable of eliciting immune responses. Such anti-idiotypic antibodies to maternal cells or antibodies generated against trophoblast alloantigens during pregnancy have been postulated to down regulate maternal rejection responses in successful pregnancies (Saji, 1993). Evidence in support of this contention has come from murine studies of the

resoption prone CBA/J X DBA/2 mating combination. Chaouat and Lankar in 1988 showed that injection of pregnant CBA/J females, before day 6 of gestation, with anti-idiotypes to the maternal cells and antibodies coded to react to BALB/c lymphocytes, reduced the high resorption rate. Although they went on to suggest that anti-idiotypes may be responsible for the beneficial effects of leukocyte alloimmunization, this was later contradicted by studies showing that the effects of leukocyte alloimmunization in mice are most likely due to non-specific immunomodulation rather than specific antibodies (Baines *et al.*, 1996). Other researchers have also attributed the benefit conferred by IvIg for the treatment of antiphospholipid antibody syndrome (Caccavo et al., 1994) and spontaneous recurrent abortion, to the presence of anti-idiotypes in the IvIg (Torry *et al.*, 1997). Nevertheless, confounding the role of "protective antibodies" during successful reproduction, IvIg treatment, and leukocyte alloimmunization are the normal pregnancies observed in women with agammaglobulinemia.

5. Decidual Effectors and Early Embryo Loss in Resorption-Prone Murine Matings

(a) Role of Macrophages

As elements of the innate immune system that play a critical role in host defense, macrophages are an excellent illustration of a cell population that is prominently diversified in its functions. These migratory cells are the terminally differentiated components of the bone marrow derived mononuclear phagocyte system and are present in most tissues of the body. The activation of macrophages results from the differentiation of blood monocytes into tissue specific effector cells that have the potential to carry out some function that can't be executed by the resting precursors. In

terms of innate or nonspecific immunity, macrophages have been described as "scavenger cells" attempting to directly clear foreign antigens, pathogens, and apoptotic bodies by phagocytosing them into intracellular vesicles and exposing them to lysosomal enzyme degradation. Macrophage cytotoxicity also correlates with their capacity to secrete *in situ* reactive oxygen intermediates (ROI), reactive nitrogen intermediates (RNI), and a myriad of enzymes (Crawford *et al.*, 1994). Systemically, activated macrophages produce cytokines that stimulate inflammation and fever. In the context of specific host defense, macrophages may also present processed antigen on their surface in association with MHC class II for the purpose of educating and activating T lymphocytes and the adaptive immune response.

Although murine studies have revealed the presence of macrophages in both cycling non-pregnant uteri and implanted gestating uteri (Hunt and Robertson, 1996; Platt and Hunt 1998), their functions in these milieux have yet to be determined (Hunt *et al.*, 1997b). Following implantation, macrophages have been shown to increase in density (Hunt 1994), and express MHC class II molecules on their surface (Hunt *et al.*, 1985). Although MHC class II expression is an indicator of macrophage activation, antigen processing and presenting by these phagocytes *in situ* has not been clearly documented (Clark, 1993). Furthermore, decidual macrophages are diminished in their ability to mount DTH responses, best illustrated by exacerbated local intracellular *Listeria monocytogenes* infections during murine pregnancy (Redline and Lu, 1988). Overall, suppression of the macrophage effector functions described above may be induced by the decidual environment in order to protect the developing conceptus from immune rejection. At the same time, however, uterine macrophages may be intricately involved

in establishing local immunosuppression by secreting TGF β for autocrine down regulation (Crawford *et al.*, 1994). They may promote placental growth by secreting CSF-1 (Hill, 1992) or promote healthy vascularization of the implantation sites by producing the smooth muscle relaxant, nitric oxide (NO) (Hunt *et al.*, 1997b). Finally, the markedly increased incidence of pregnancy loss in macrophage and CSF-1 deficient *op/op* "osteopetrotic" mice, further supports the assertion that macrophages make important contributions to successful pregnancy (Pollard *et al.*, 1991).

As mentioned in the beginning of this review, various lines of evidence have implicated a macrophage involvement in murine early embryo loss. First, Duclos and colleagues in 1994 and 1995 demonstrated that treatment of gravid DBA/2-mated CBA/J female mice (a high loss mating) with rat anti-macrophage anti-sera (anti-Mac-1 antibody) significantly reduced the incidence of early embryo resorption. Next, by employing immunohistochemistry with macrophage-specific cell surface markers (Mac-1, F4/80), the same investigators showed that decidual infiltration by macrophages was an important early event preceding embryonic resorption. Specifically, by day 8 of gestation, well before any obvious embryo destruction was evident, approximately 20-30% of the implantation sites from DBA/2-mated CBA/J female mice had significantly elevated numbers of infiltrating macrophages (Duclos *et al.*, 1995; Baines *et al.*, 1997). Interestingly, this percentage corresponded to the natural resorption rate observed in this murine mating model at day 12 of gestation. Also noted was the observation that "high loss matings" (20-30%) had a greater proportion of embryos with significantly elevated numbers of decidual macrophages than "low loss matings" (5-10%).

By using molecular technologies to detect the expression of proto-oncogenic cellular activation markers (Duclos *et al.*, 1996) and immunohistochemistry to detect MHC class II (Duclos *et al.*, 1995), it has been demonstrated that at day 8 of gestation, 20-30% of (CBA/J X DBA/2) F1 implantation sites display significantly elevated levels of macrophage activation. Indeed, an analysis of resorbing embryos at day 8 of gestation has shown evidence of primed inflammatory macrophages in the decidua. *In vitro* incubation of these cells with LPS (a macrophage triggering agent) was shown to induce the production of the cytotoxin effectors, nitric oxide (NO) and TNFα (Haddad *et al.*, 1995; Gendron *et al.*, 1990). Furthermore, genetic studies have indirectly substantiated a role for interferon gamma (IFNγ) in the priming of decidual macrophages by demonstrating the simultaneously increased expression of IFNγ, TNFα, and inducible nitric oxide synthase (iNOS) mRNA in 20-30% of CBA/J X DBA/2 F1 implantation sites before overt embryo pathology is apparent (Haddad *et al.*, 1997a).

(i) TNFα

The macrophage activation marker Tumor necrosis factor alpha (TNFα) is a TH1 cytokine that exerts pleiomorphic effects on many different cell types. Originally described for its cytotoxic and cytostatic effects on tumor cells, it is also an important factor in: inflammatory processes, cellular growth and differentiation, initiating apoptosis, the host response against gram negative bacteria, and many other activities (Sidhu and Bollon, 1993; Rabaye *et al.*, 1991; Vassali, 1992). Activated macrophages are the major sources of this 17-kd polypeptide mediator but natural killer cells and T cells have been shown to produce it upon activation (Peters *et al.*, 1986; Hill, 1992;

Haddad *et al.*, 1997). Uterine and trophoblast cells in humans (Chen *et al.*, 1991; Vince *et al.*, 1992) and in rodents (Yelavarthi *et al.*, 1991) have also demonstrated TNFα production. Likewise, TNFα cell surface receptor I and II mRNAs have been localized in murine pregnant uteri and placentae (Roby *et al.*, 1995). Since TNFα expression has been reported as cycle and gestational stage related (Roby *et al.*, 1994; Hunt *et al.*, 1996), it has been proposed that it is involved in the normal processes of pregnancy, perhaps by regulating trophoblast growth and differentiation, or by stimulating genes involved in tissue remodeling and apoptosis ((Chen *et al.*, 1991; Hunt *et al.*, 1996).

Even though TNFα appears to play a pivotal role in regulating reproduction and embryonic development, numerous findings demonstrate its involvement in the pathways leading to premature pregnancy failure. For instance, administration of TNFα raises the rate of resorption in various murine matings (Chaouat *et al.*, 1990). Alternatively, soluble receptors neutralizing TNFα (Arck *et al.*, 1997), anti-TNFα antibodies, and pentoxifylline (TNFα suppressor) block early embryonic demise (Gendron *et al.*, 1990). Pooled placentas from "high loss" CBA/J X DBA/2 matings display significantly enhanced levels of TNF mRNA that are not observed in "low loss" CBA/J X BALB/c matings (Tangri and Raghupathy, 1993). It has also been proposed that LPS induced embryo abortion may be due to LPS triggering of TNFα production by primed decidual macrophages (Gendron *et al.*, 1990). Studies employing *in situ* hybridization localize both TNFα and TNFα receptor I mRNA to the fetomaternal interface and detect substantially stronger hybridization signals in "high abortion" placentas than in control "low abortion" placentas (Gorivodsky *et al.*, 1998). Finally, the benefit conferred to high

loss murine matings by alloimmunization has recently been shown to correlate with a decline in TNFα expression at the fetomaternal interface (Gorivodsky *et al.*, 1998).

(ii) Nitric Oxide

NO, a reactive nitrogen intermediate that exhibits diverse physiological effects, is produced by a heterogeneous assortment of cells. (Moncada *et al.*, 1991). In the context of the immune response, macrophage activation by LPS, IL-1, IFNγ, or TNFα (Ding *et al.*, 1988) has been shown to induce the synthesis of NO from L-arginine by the inducible isoform of nitric oxide synthase (iNOS) (Knowles and Moncada, 1994). Since injection of pregnant mice with LPS, IFNγ, and TNFα leads to embryo resorption, and treatment with a selective inhibitor of iNOS (Aminoguanidine) reduces the incidence of embryo resorption in CBA/J X DBA/2 matings, macrophage-derived NO cytotoxin has been indicted as a molecular effector of early embryo loss (Haddad *et al.*, 1995). Supporting this contention is the finding that LPS stimulated decidual cells from visibly resorbing embryos displayed about 5-10 times greater levels of NO production than non-resorbing embryos (Haddad *et al.*, 1995). Significantly, immunohistochemical analysis has also revealed the presence of decidual cells coexpressing iNOS and Mac-1. Gene expression analysis further substantiates a role for NO in early embryo loss by showing elevated levels of iNOS message in 20-30% of CBA/J X DBA/2 embryo implants before embryo pathology ensues (Haddad *et al.*, 1997b).

(b) Natural Killer Cells

As components of natural immunity that do not require prior priming or activation

to exert cytotoxicity, natural killer cells are thought to constitute the first line of defense against virally infected cells, graft cells, and transformed cells (Trinchieri *et al.*, 1989). Lacking cell surface idiotypes such as immunoglobulins found on B cells and TCRs on T cells, these phenotypically distinct large granular cells also distinguish themselves from other lymphocytes by their ability to detect "non self MHC" on target tissues (Yokoyama, 1997). Generally, ubiquitously expressed autologous MHC class I molecules block NK mediated cytoysis by binding to NK surface inhibitory receptors. For example, the LY-49 family of murine NK receptors are type II transmembrane C-lectin like proteins that have been shown to transmit inhibitory signals that impede NK activation, cytotoxicity, and cytokine production (Ortaldo *et al.*, 1997). Likewise, killer inhibitory receptors (KIR) in humans, belonging to the immunoglobulin superfamily of molecules, have also been shown to inhibit NK effector functions upon receptor engagement with MHC class I ligands (Lanier and Phillips, 1996; Carosella *et al.*, 1999).

Given that detection of "missing self" liberates NK cells from a state of inhibition, it would be reasonable to expect NK mediate cytolysis and destruction of placental trophoblasts, as they are deficient of polymorphic MHC class I molecules. Yet, *in vitro* studies have indicated that trophoblasts are resistant to killing by conventional NK cells (Zuckermann and Head, 1988). Recent work in humans, has focused on a novel, non-classical, non-poymorphic MHC class I molecule, called HLA-G, as a possible regulator of NK cells during pregnancy (Rouas-Freiss *et al.*, 1997). Human leukocyte antigen-G expressed by trophoblasts, has been postulated to inhibit NK-mediate cytolysis by binding to KIR's, and recent discovery of an HLA-G-specific KIR on all NK cells has supported this contention (Rajagopalan and Long, 1999).

Various findings have implicated the involvement of NK cells in early embryo loss. In humans, elevated numbers of blood and decidual CD56+ NK cells have been noted in women spontaneously aborting karyotypically normal embryos (Lachapelle *et al.*, 1996; Vassilidou and Bulmer, 1996). In mice, modulation of NK activity has been shown to affect the incidence of embryo resorption. For instance, injection with poly I:C, a double stranded synthetic RNA that boosts NK activity and production of IFNγ, significantly increases the resorption rate (deFourgerolles and Baines, 1987; Kinsky *et al.*, 1990) both in abortion prone DBA/2-mated CBA/J females as well as in low loss mating combinations. Alternatively, treatment of mice with anti-sera raised against the NK marker asialo GM1, has been shown to abrogate NK functions and profoundly reduce the rate of resorption (deFourgerolles and Baines, 1987). Furthermore, immunohistochemistry has demonstrated an NK involvement in the events preceding early embryo loss, as 20-30% of F1 CBA/J X DBA/2 embryos at day 8 of gestation displayed significantly increased infiltration of asialo GM1+ cells. (Gendron and Baines, 1988).

(i) Role of IFNγ

Although lymphokine activated killer (LAK) cells have been shown to effectively kill murine trophoblasts *in vitro* (Drake and Head, 1989), early embryo resorption has not been associated with direct NK-mediated cytotoxicity (Baines *et al.*, 1997). Apart from perforin and granzyme-mediated cytolysis, TNFα induced apoptosis, and antibody dependent cellular cytotoxicity (ADCC), NK cells can also exert their effects by producing cytokines. During early embryo loss, NK production of IFNγ may

prime decidual macrophages to an activated state (Baines *et al.*, 1997). Indeed, administration of exogenous IFNγ to pregnant female mice has been shown to compromise the outcome of pregnancy (Chaouat *et al.*, 1990), whereas injection of rat anti-mouse IFNγ anti-sera significantly reduces embryo demise (Haddad *et al.*, 1995). Furthermore, IFNγ-deficient GKO mice have demonstrated a resistance to LPS-induced pregnancy loss (Haddad *et al.*, 1997a). LPS is thought to function synergistically with IFNγ in a multi-step process to induce the production of macrophage effectors TNFα and iNOS and subsequent early embryo loss.

(c) Granulated Uterine Lymphocytes

Large granulated lymphocytes have been shown to transiently populate the uterine mesometrial triangle in early murine and human pregnancy, following decidualization (Whitelaw and Croy, 1996; King *et al.*, 1997). Phenotypically characterized as NK lineage cells (Zheng *et al.*, 1991; Croy and Kiso, 1993), proliferation, differentiation, and synthesis of iNOS and TNFα by these bone marrow-derived cells appears to be under the control of steroid hormones (Hunt *et al.*, 1997a) and may play important roles in normal gestation. In human, approximately 70% of decidual leukocytes have been characterized as $CD56^{bright}$ $CD16^{-}$ $CD3^{-}$ granulated NK cells (King and Loke, 1991; King *et al.*, 1997). In rodents, granulated NK-like cells have been shown to reside in a specialized uterine tissue called the metrial gland (Liu *et al.*, 1994). Granulated metrial gland cells (GMG) bear NK surface markers asialo GM1 and LGL-1, lack B and T cell and macrophage specific markers (King *et al.*, 1997), and have been shown to contain cytoplasmic granules filled with cytolytic perforin and granzyme molecules (Parr *et al.*, 1990).

Similar to conventional NK cells, GMG cells have been shown to exhibit cytotoxic activity against Yac-1 targets *in vitro* following the addition of IL-2 (Croy *et al.*, 1991; King *et al.*, 1997).

Although a number of postulates have been proposed to explain the roles played by GMG cells during normal pregnancy on the large part this remains to determined. GMG secretion of immune mediators has been suggested to control trophoblast migration and placentation (King *et al.*, 1997). Furthermore, recent demonstration of iNOS mRNA positive GMG cells has lead to the suggestion that NO produced by GMG cells may be important in mediating healthy vascularization of implantation sites (Hunt *et al.*, 1997b; Guimond *et al.*, 1998). The most convincing evidence implicating the importance of NK-like GMG cells during gestation is based on studies of NK-deficient or gene-ablated mice (Croy *et al.*, 1997). Specifically, an inability to form a metrial gland is thought to explain the exacerbated decidual pathologies, increased fetal losses, and reduced placental sizes that are associated with NK-deficient transgenic Tg epsilon 26 mice (Guimond *et al.*, 1997; 1998).

Since asialo GM1+ cells have been directly associated with early embryo loss in DBA/2-mated CBA/J mice, a GMG involvement has been proposed. However, both immunohistochemical examination and *in situ* hybridization of day 9 utero-placental units have indicated that the distribution of perforin positive GMG cells is essentially the same near resorbing and non resorbing CBA/J X DBA/2 F1 embryos (Zheng *et al.*, 1993). Moreover, anti-asialo GM1 treatment has not been shown to significantly deplete GMG cells even though treatment reduces resorption rates in high loss mating models (Parr *et al.*, 1987; Arck and Clark, 1997).

(d) TH1:TH2/3 Balance

TH1 and TH2/3 cells are subsets of CD4+ T helper cells that display very distinct cytokine profiles. In general, TH1 cytokines are associated with inflammatory and cell-mediated responses that usually predominate during infections by intracellular pathogens. TH1 cytokines include IFNγ, TNFα, and IL-2. On the other hand, anti-inflammatory TH2/3 cytokines tend to bias host responses away from cell-mediated immunity, towards specific humoral immunity. TH2/3 cytokines include IL-4, IL-5, IL-10, and IL-13. Considering that normal pregnancy constitutes a TH2/3 type of response (Wegmann *et al.*, 1993; Raghupathy, 1997) it has been postulated that early embryo loss is a consequence of increased TH1 reactivity at the fetomaternal interface and a reduction of TH2/3 cytokines. As mentioned previously, TH1 cytokines such as TNFα and IL-2 have been shown to compromise the outcome of pregnancy whereas TH2/3 IL-10 prevents early embryo demise (Krishnan *et al.*, 1996). Furthermore, mixed lymphocyte-placenta reactions (MLPR) have demonstrated that placenta from resorption-prone CBA/J X DBA/2 matings stimulate lymphocyte production of IFNγ, TNFα, and IL-2 to a greater extent than low loss matings (Tangri *et al.*, 1994).

(e) γδ T Lymphocytes

Lacking conventional T cell markers such as CD4 and CD8, γδ T cells are innate lymphocytes that express an alternative TCR in association with the CD3 complex. γδ T cells are scarce in the blood and their residence in the epithelium of skin and mucosal surfaces, and ability to produce cytokines has been shown to provide a first line of

defense against various microorganisms (Born *et al.*, 1990; Arck *et al.*, 1999). γδ T cells represent approximately 60% of the small number of T cells detected in the uterus and have recently been shown to function as immunosuppressor cells during murine gestation by producing TGFβ2 (Clark *et al.*, 1997). Since they have been shown to produce cytokines that alter the TH1:TH2/3 balance at the fetomaternal interface, and may recognize trophoblast cells that express heat shock proteins (HSP), decidua-associated γδ T cells have also been implicated as effectors determining the outcome of pregnancy.

Rationale and Objectives of the Study

The maternal immune system appears to exert a key role in determining the outcome of mammalian pregnancy. Immunohistochemical studies of pregnant uteri have revealed the presence of various populations of immune cells during implantation, normal gestation, and premature pregnancy loss (Saito *et al.*, 1993; Gendron and Baines, 1988; Duclos *et al.*, 1996). While anti-inflammatory TH2/3 cytokines are expressed during normal pregnancy, numerous investigations have correlated pregnancy failure with inflammatory TH1 cytokines (Wegmann *et al.*, 1993; Krishnan *et al.*, 1996). In humans, approximately 25% of normal embryos undergo spontaneous early abortion by the third week of gestation (Edmonds *et al.*, 1982; Lippman and Farookhi, 1986; Wilcox *et al.*, 1988). Since murine models provide a practical approach to studying pregnancy, this study uses the high loss CBA/J X DBA/2 mating pair to investigate the immunological cellular and molecular factors associated with implantation and early embryo loss. This

experimental mating model has a natural resorption rate of 20-30% by day 12 of gestation (Clark *et al.*, 1980).

Although the etiologies and mechanisms of spontaneous abortion and early embryo loss in most species remain to be fully determined, early embryo resorption in DBA/2-mated CBA/J female mice is thought to primarily involve the non-specific arm of the immune response. A conceptual model of the role of decidual macrophages, NK cells, and inflammatory cytokines in the rejection of allogeneic fetoplacental units has been described and is the rationale behind this study (Baines *et al.*, 1997). It has been hypothesized that an undetermined stimulus activates resting NK cells to infiltrate the maternal deciduum and produce pro-inflammatory cytokines that affect the activity of decidual macrophages. NK production of IFNγ may act as a priming signal of decidual macrophages while subsequent NK production of TNFα may trigger the release of cytolytic effectors by the activated macrophages. Since administration of bacterial LPS to gravid mice has been shown to cause the majority of embryos to resorb, it has also been proposed to act synergistically with IFNγ to trigger the release of macrophage-derived cytotoxic effector factors (TNFα, NO). Consequently, the release of increased levels of cytolytic TNFα and NO may cause damage to placental trophoblast cells or other fetoplacental targets resulting in embryo death and resorption. Significantly, recent studies have indicated that the role of macrophage effectors may not be to directly damage the trophoblast itself, as trophoblasts are resistant to killing by TNFα, NK cells and macrophages, but to induce ischemia, thrombosis and necrosis in the vasculature that surround the conceptus (Clark *et al.*, 1998).

In this study, gene expression analyses were undertaken to investigate the earliest events in the activation of decidual NK cells and macrophages in initiating early embryo loss. Gene expression analysis is a sensitive technique that detects the presence and state of activation of cells by quantifying the expression of unique genes. This study attempts to acquire information on the primary causative factors of early embryo loss and considers IFNγ and perforin mRNA to be molecular markers of decidual NK cells and TNFα and iNOS mRNA as macrophage activation markers.

As it has been proposed that resorption is preceded and caused by an increased decidual infiltration of inflammatory cells, the first aim of this study is to examine at the molecular level, the earliest presence of activated NK cells and macrophages in decidual tissues (day 6-8). Since abnormal production of inflammatory cytokines may be associated with pregnancy failure, a second objective is to investigate and detect the earliest expression of IFNγ, perforin, TNFα and iNOS mRNA as a correlate with early embryo loss in individual CBA/J X DBA/2 embryo implantation sites. Finally, as T lymphocytes are also major producers of IFNγ (Young *et al.*, 1995) a third objective of this study is to obtain evidence that supports the view of decidual NK cells as the sole or major sources of IFNγ in resorption prone CBA/J X DBA/2 placentae.

MATERIALS AND METHODS

1. Mice and Matings

CBA/J female mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) at 8 to 10 weeks of age and DBA/2 male mice were obtained from Charles River (St. Constant, Quebec, Canada). The mice were housed and handled according to the guidelines of the Canadian Council for Animal Care. In brief, mice were maintained in standard open-top wire cages with free access to food and water. The specific pathogen free facility was illuminated by a 12-hour light-dark cycle. The animals were acclimatized to the animal care facilities for at least 6 weeks before mating commenced. Pregnancies were obtained by overnight cohabitation of four estrus CBA/J female mice housed with one DBA/2 male mouse and checked daily for the appearance of a copulatory plug. The morning of the sighting of the vaginal plug was arbitrarily defined as day 0 of pregnancy.

2. Acquisition of Tissues

Pregnant CBA/J female mice were sacrificed by cervical dislocation at various stages of gestation, usually from day 6 to day 12. At day 7 and 8, individual embryos together with the implantation sites were harvested and stored at -80°C for analysis by reverse transcriptase polymerase chain reaction (RT-PCR). At day 6, due to difficulty in separating tissues, individual embryos, implantation sites and uterine wall were used as sources of total RNA in the RT-PCR analysis. Furthermore, uteri from non-pregnant CBA/J mice and non-implanted portions of pregnant uteri at days 6-8 of gestation served

as tissue controls and were analyzed by RT-PCR to compare the basal tissue activities to the events occurring at the implantation sites.

3. Total RNA Extraction

Trizol Reagent (Life Technologies, Burlington, Ontario, Canada) was used to isolate total RNA from the tissues. This RNA isolation procedure is based on the method developed by Chomczynski and Sacchi, 1987. Briefly, the tissues were placed in 1 ml of Trizol Reagent, homogenized with a Tissuemizer homogenizer (Tekmar, Cincinnati, OH, USA) and then incubated at room temperature for 5 minutes to enable the complete dissociation of nucleoprotein complexes. The homogenized samples were then mixed with 0.2ml of chloroform, shaken vigorously, and incubated at room temperature for an additional 2-3 minutes to remove lipids. Following the incubation, a centrifugation at 10,000 X g for 15 minutes at 4°C gave rise to three phases: a lower red phenol chloroform phase, an interface, and a RNA containing colorless aqueous phase. The aqueous phase was then isolated, mixed with 0.5ml of isopropanol, and incubated at minus 20°C overnight to precipitate the RNA. On the following day, the samples were centrifuged once more at 10,000 X g for 10 minutes at 4°C to isolate the RNA pellet. The RNA pellets were then washed once by adding 1ml of 75% ethanol and centrifuging at 5,000 X g for 5 minutes at 4°. Finally, the RNA pellets were air dried and redissolved in RNAse-free water (0.1% of diethyl pyrocarbonate in distilled water) by incubating the samples at 60°C for 10 minutes. The RNA concentration and purity was calculated from spectrophotometric readings at 260nm and 280nm. The RNA was occasionally separated

by electrophoresis in a 1% agarose gel in which the integrity of the 28s and 35s ribosomal RNA bands was used as an indicator of the quality of the extracted RNA.

4. DNAse Treatment

Total RNA was treated with Deoxyribonuclease I, Amplification Grade (Life Technologies) to eliminate any traces of genomic DNA. The reaction was prepared from 2 μg of total RNA, 1μl of 10x DNAse I reaction buffer (Life Technologies), 1μl of DNAse I, and enough DEPC-treated water (1ml of diethyl pyrocarbonate in 1L of distilled water) to give a total volume of 10μl. The reaction was incubated at room temperature for 15 minutes and then terminated by the addition of 1μl of 25mM EDTA.

5. RT-PCR Analysis of IFNγ, iNOS, TNFα, Perforin, and G6PDH mRNA at Days 6-8 of Pregnancy

The DNAse treated total RNA was reversed transcribed to cDNA using a method adapted from that previously described by Haddad *et al.*, 1997. Briefly, the reaction was prepared from 1μl of 0.1M DTT (dithiothriotol), 4μl of 5x first strand buffer (Life Technologies), 2μl of 5mM dNTP mix (Pharmacia, Baie d'Urfe, Quebec, Canada), 1μl of 100nM random hexamer primers (Life Technologies), 1μl of 40units/μl RNAse Inhibitor (Pharmacia, Baie d'Urfe, Quebec, Canada), 1μl containing 200units of Moloney Murine Leukemia Virus Reverse Transcriptase (Life Technologies). The 10μl DNAse treated RNA preparation was incubated at 65°C for 10 minutes and then added to the reaction mixture to give a total volume of 20μl. A one-hour incubation at 37°C was allowed for cDNA synthesis. The cDNA was then diluted 1:8 with sterile water, heated to 95°C for

10 minutes, and stored at -20°C. Each experiment included a negative RT control in which no reverse transcriptase was added to an equal quantity of RNA.

Following RT, PCR was conducted in a total volume of 50µl. The $MgCl_2$ concentration, cDNA concentration, and the number of cycles were standardized to optimize the reaction for each sequence and primer pair and to ensure that logarithmic amplification was not run to saturation levels. Five microliters of the cDNA synthesized in the above reaction was used to detect G6PDH, and perforin mRNA. Ten microliters of cDNA was used to detect IFNγ, iNOS, and TNFα mRNA. The cDNA was added to 5µl of 10x PCR buffer (50mM KCl, 10mM TrisHCl, 1.5mM $MgCl_2$, 0.01% Triton X), 1µl of 0.15µg/µl of both 5' and 3' cytokine specific primers, 1µl of 5mM dNTP mix (Pharmacia, Baie d'Urfe, Quebec, Canada), 1µl of 2.2units/µl of Taq Polymerase (Life Technologies) and sterile water. The mixture was heated to 95°C for 4 minutes and then PCR was performed according to the following protocol: 1 minute of denaturation at 94°C, 1.5 minutes at the appropriate annealing temperature, 1 minute of extension at 72°C, for 30 cycles. Following the PCR there was a final extension for 10 minutes at 72°C. Each PCR experiment included a negative control reaction in which no cDNA was added.

6. Southern Blotting

The PCR amplified products were resolved on a 1% agarose gel that contained ethidium bromide and were visualized by ultraviolet light. Following photo-documentation, the gel was first immersed in a denaturation buffer (87.66g NaCl, 20g NaOH in 1000ml of distilled water) for 30 minutes and then in a neutralization buffer (87.66g NaCl, 60.5g Trisma Base in 1000ml of distilled water pH 7.5) for an additional

30 minutes. The DNA was then transferred by capillary blotting onto a nylon membrane (Hybond-N, Amersham Life Science) according to the method of Southern (Southern, 1975). A UV Stratalinker (Stratagene) was used to crosslink and immobilize the DNA on the membranes which were then stored dry at room temperature.

7. 5' End Labeling of Oligonucleotide Probes

The "Ready To Go T4" polynucleotide kinase kit (Pharmacia) was used to radiolabel the 5' end of cytokine specific oligonucleotide probes. Briefly, 10 pmoles of probe, 1µl of 10 µCi/µl [γ^{32}-P]ATP (Mendel) and 25µl of sterile water were added to a tube of reconstituted "Ready To Go" T4 polynucleotide kinase. The reaction was incubated at 37°C for 30 minutes and then terminated by the addition of 5µl of 250mM EDTA and 100µl of TE buffer. The mixture was then purified through a G50 Sephadex mini-column which was spun at 3,000 X g to remove unbound [γ^{32}-P]ATP and stored at -20°C.

8. Hybridization with Gene Specific Internal Probes

The hybridization was done using a method adapted from that previously described by Haddad *et al.*, 1997b. Briefly, the membranes were immersed for 2 hours in 30ml of a prehybridization buffer containing 7.5ml of 20x SSC (88.23g of Tri-sodium citrate, 175.32g of NaCl in 1000ml of water, pH7.5), 3ml of 100% Denhardt's solution (2.0g bovine serum albumin, 2.0g of Ficoll 400, 2.0g of polyvinylpyrrolivolone in 100ml of water), 10.5ml of 20% SDS, 5.9ml of water and 100µl of salmon sperm DNA (Pharmacia). Radiolabeled oligonucleotide was then added to the solution and the

membranes were further incubated overnight either at 42°C or 65°C, depending on the oligo melting temperature. The membranes were then thoroughly washed 4 times with buffers containing varying concentrations of SSC and SDS. Following the last wash, the membranes were wrapped in plastic film and exposed to a phosphoimaging screen or autoradiographic film. The desired maximum density of the G6PDH bands (G6PDH is a constitutively active housekeeping gene that was used to normalize the expression of IFNγ, perforin, TNFα and iNOS mRNA) was within the range of $10^5 - 10^6$ units. Bands on the phosphoimaging cassette were visualized with a Molecular Dynamics phosphoimager (San Diego, CA) and analyzed with the ImageQuant program. Bands on autoradiographic films were analyzed with a scanning densitometer.

9. Statistics

Microsoft Excel and SPSS software were used to perform statistical analysis of data. The normality of the sample distribution was analyzed with statistics that measure central tendancy (mean, median) and the null hypothesis was rejected when the ratio of skewness (asymmetry of the sample distribution) to its standard error was determined as ≥ 2. Intragroup K means were then computed by SPSS software (SPSS Inc. Chicago, IL) where bimodal distributions of data were apparent. The upper 95% confidence limits of normal values were defined as, K mean of cluster 1 + 1.96 standard deviates.

10. Designing Primers and Probes

Primer sets and internal oligonucleotide probes were synthesized at the Sheldon Biotechnology Center (Montreal, Quebec, Canada). The sequences for G6PDH, iNOS,

TNFα, were obtained from previously published papers (Haddad *et al.*, 1997b). The primer and probe sequences for IFNγ and perforin were designed with the program Oligo 4S using the genbank cDNA sequence. Sequences were discriminated according to the following criteria: GC content, length, self annealing, primer dimerization, gene homology, positioning, melting temperature, product size, spanning of introns. Oligonucleotides were stored as concentrated stocks of 1μg/μl at -20°C.

11. Preparation of Decidual and Spleen Cell Suspensions

Uterus and spleen were removed from pregnant CBA/J mice at day 9 of gestation and the individual embryos and implantation sites were pooled together to increase the cell yield. Cell suspensions were prepared in Dulbecco's PBS (0.1g/L $MgCL_2$-H_2O, 0.2g/L KCl, 0.2g/L KH_2PO_4, 1.15g/L Na_2HPO_4, 8.0g/L NaCl, 0.133g/L $CaCl_2$-$2H_2O$) supplemented with 2.5% FCS. Embryo-placental units were placed in 5ml of the balanced salt solution, chopped into small pieces with a sterile blade and drawn up and down several times in a 21.5G needle to further disperse the cells. Spleen cell suspensions were prepared by homogenizing the tissue in 5ml of balanced salt solution with a steel screen and a sterilized glass tube. Both decidual and spleen cell suspensions were then incubated on ice for 3 minutes to sediment any debris and mixed with 12ml of ACK lysing buffer (0.15M Ammonium Chloride, 10mM Potassium Bicarbonate, 0.1mM Sodium EDTA, pH 7.3) for 3 minutes at room temperature to disrupt the red blood cells. The cells were then washed 3 times by resuspending the pellet in Dulbecco's PBS supplemented with 2.5% FCS and centrifuging at 300 X g for 10 minutes. Following the

last wash, a viable cell count was done using trypan blue and the cells were resuspended at a concentration of 2 X 10^7 cells/ml in balanced salt solution.

12. Immunofluorescent Staining of Decidual and Spleen Cells with Phycoerythrin-Conjugated Rat Anti-Mouse Pan NK Cell Monoclonal Antibody

PE conjugated rat anti-mouse pan NK cell monoclonal antibody DX5 (Pharmingen, Mississauga, Ontario) was used to label cells from the deciduum and spleen that express the DX5 natural killer cell surface marker. Briefly, 50µl aliquots of the 2 X 10^7cells/ml suspension were mixed with 0.25µg of Fc Block (Pharmingen) and incubated on ice for 5 minutes. Fc Block is a rat anti-mouse CD16/CD32 monoclonal antibody that blocks the nonspecific binding of the rat immunoglobulins to the FcγII receptors on mouse B lymphocytes. One microgram of 0.2µg/µl PE-linked rat anti-mouse pan NK cell monoclonal antibody was then mixed with 45µl of Dulbecco's PBS supplemented with 2.5% FCS and added to the cell suspension. The reaction was incubated on ice for 15 minutes in the dark, washed three times with Dulbecco's PBS supplemented with 2.5% FCS, and centrifuged at 350 X g for 10 minutes. Following the last wash, the cells were resuspended in 500µl of wash buffer and analyzed by flow cytometry. Each labeling experiment included a negative control in which no anti-DX5 antibody was added. Finally, a FACS/SORT was performed to separate the DX5 negative cells from the positively stained cells.

13. RT-PCR Detection of IFNγ mRNA in Decidual and Spleen Cells

Total RNA minipreps from the sorted cell populations were performed with the

RNeasy Mini Kit (Qiagen, Santa Clarita, CA, USA). Briefly, the pure cell populations were first lysed in the presence of a guanidinium isothiocyanate containing buffer and then homogenized using a QIAshredder spin column (Qiagen). Following the addition of one volume of 70% ethanol, the lysate was applied onto a RNeasy mini spin column and centrifuged at 16,000 RPM for 15 seconds. The total RNA that remained bound to the silica-gel-based membrane present in the spin column and was finally eluted in 30μl of RNase free water and stored at -80°C.

Total RNA isolated from the DX5 positive, DX5 negative cells was then treated with Amplification Grade Deoxyribonuclease I (Life Technologies) according to the method described above. The reaction was prepared from 15μl of total RNA, 2μl of DNAse I, and sufficient RNAse free water to give a total volume of 20μl. Reverse transcription of DNAse treated RNA to cDNA was done by mixing 10μl of RNA with 4μl of 5x first strand buffer (Life Technologies), 1μl of 0.1M DTT, 2μl of 5mM dNTP mix (Pharmacia, Baie d'Urfe, Quebec, Canada), 1μl of 40units/μl RNAse Inhibitor (Pharmacia, Baie d'Urfe, Quebec, Canada), 1μl of 100nM random hexamer primers (Life Technologies), and 1μl of 200units/μl Moloney Murine Leukemia Virus Reverse Transcriptase (Life Technologies) and incubating at 37°C for one hour. Each RNA sample included a negative RT control in which no reverse transcriptase was added.

PCR detection of IFNγ mRNA in the DX5 positively and negatively expressing cell populations was done in a reaction volume of 50μl. Five microliters of the undiluted cDNA was added to 5μl of 10x PCR buffer (containing 1.5mM $MgCl_2$), 1μl of 0.15μg/μl of both 5' and 3' cytokine specific primers, 1μl of 5mM dNTP mix, 1μl of 2.2 units/μl of Taq Polymerase, 1μl of 0.25% polyacrilamide and 35μl of sterile water. Forty-five

cycles of PCR was performed according to the protocol described above. Each experiment included a negative control PCR reaction in which no cDNA was added.

The PCR products were electrophoretically separated in a 1% agarose gel that contained 10mg/L ethidium bromide and were subsequently visualized by UV light and photo-documented.

Table 1. Primer and Probe Sequences, Primer Annealing Temperature and Expected PCR Product Size.

GENE	Oligonucleotide Sequence listed from 5' to 3'	Annealing Temperature	Expected PCR Product Size in bps.
G6PDH **Sense** **Antisense** **probe**	CTA AAC TCA GAA AAC ATC ATG GC GTG TAT CAG CTT GGT GGA AGG GAG CAG GTG GCC CTG AGC CG	58°C	111
IFNγ **Sense** **Anti-sense** **probe**	ACA CTG CAT CTT GGC TTT GC CGA CTC CTT TTC CGC TTC CT GGA GGA ACT GGC AAA AGG ATG G	60°C	426
PERFORIN **Sense** **Anti-sense** **Probe**	TTT TCC TGC TGC TGC CAC GAC CTG GCC GTG ATA AAG TGC GTG CCA TAG ACA GAG GGT GCA GGT GCG GTC AGG	60°C	646
INOS **sense** **Anti-sense** **probe**	CTT CCG AAG TTT CTG GCA GCA GCG GAG CCT CGT GGC TTT GGG CTC CTC ACG TTC AGG ACA TCC TGC AAA AGC AGC TGG	58°C	486
TNFα **Sense** **Anti-sense** **probe**	CCA GAC CCT CAC ACT CAG AT AAC ACC CAT TCC CTT CAC AG CCA AGT GGA GGA GCA GCT GGA G	58°C	498

Results

1. Experimental groups

As outlined in table 2, three CBA/J (female) X DBA/2 (male) mating groups were utilized in this study to examine the earliest events associated with activation of decidual natural killer-like cells and macrophages in initiating early embryo loss. Individual embryos along with their respective implantation sites were assayed by RT-PCR for the level of expression of specific macrophage and natural killer cell activation markers. Since early embryonic resorption in mice has been characterized as a type of post-implantation loss which is complete by approximately day 12 of gestation, and since implantation takes place at approximately day 5, the earliest gestational age included in this study was day 6. Gravid DBA/2-mated CBA/J females that were sacrificed at day 6, 7, and 8 contained an average of 7.0 ± 1.4, 9.4 ± 1.1, and 8.6 ± 1.1 implantation sites per mouse, respectively.

2. Obvious signs of early embryonic damage are not apparent at days 6-8 of gestation

Gross examination of the uterine contents of three DBA/2-mated CBA/J females sacrificed at day 12 of gestation showed that an average of 2.3 ± 0.58 implantation sites per mouse were significantly smaller, hemorrhaged and necrotic. Resorbing embryos were distinguishable at day 12 and based on an average 9.0 ± 1.0 implantation sites/mouse. This indicated a loss of 26% of the early embryos. On the other hand, gross examination of all the embryo implantation sites at days 6-8 of gestation did not demonstrate any of the obvious signs of damage observed in 26% of the CBA/J X DBA/2 F1 implantation sites at day 12. These observations are in accordance with previous studies of this high loss mating combination.

Table 2. The mating of CBA/J females with DBA/2 males.

Gestational Age in Days*	Number of Mice Assayed	Total Number of Implantation Sites
6	5	35
7	5	47
8	5	43

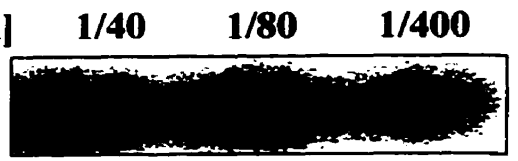
Note. Pregnant mice were sacrificed at the gestational age indicated and individual embryo implantation sites were assayed by RT-PCR for expression of macrophage and NK cell activation markers.

3. Optimization of the polymerase chain reaction

In this study, semi-quantitative PCR was employed to determine and compare the expression of natural killer cell and macrophage activation markers in individual implantation sites, relative to the level of expression of the housekeeping gene G6PDH. The amount of cDNA initially added is an important factor in this semi-quantitative assay, as it plays a critical role in determining whether the amplification reaction is linear or at saturation levels. To ensure that the PCR did not run to saturation levels, and that an increase in cDNA concentration resulted in an increased signal for the constitutively activated housekeeping gene G6PDH, PCR was performed on a series of dilutions of embryonic cDNA. As illustrated in figures 1a and 1b, a cDNA final dilution of 1/80 (equivalent to 0.025μg of total RNA) was determined as an appropriate cDNA concentration for PCR amplification of G6PDH. Notably, an increase in cDNA concentration to 1/40 resulted in an increased blot density but a slight deviation from linearity indicating the beginning of saturation of the PCR reaction. Reduction of the cDNA concentration to 1/400 resulted in a decreased blot density. Finally, a cDNA concentration of 1/80 (0.025μg of total RNA) was similarly established as appropriate for RT-PCR analysis of perforin mRNA expression and a cDNA concentration of 1/40 (0.05μg of total RNA) for RT-PCR analyses of IFNγ, TNFα, and iNOS mRNA expression.

Figure 1

A.


[cDNA]
1/40
1/80
1/400

B.

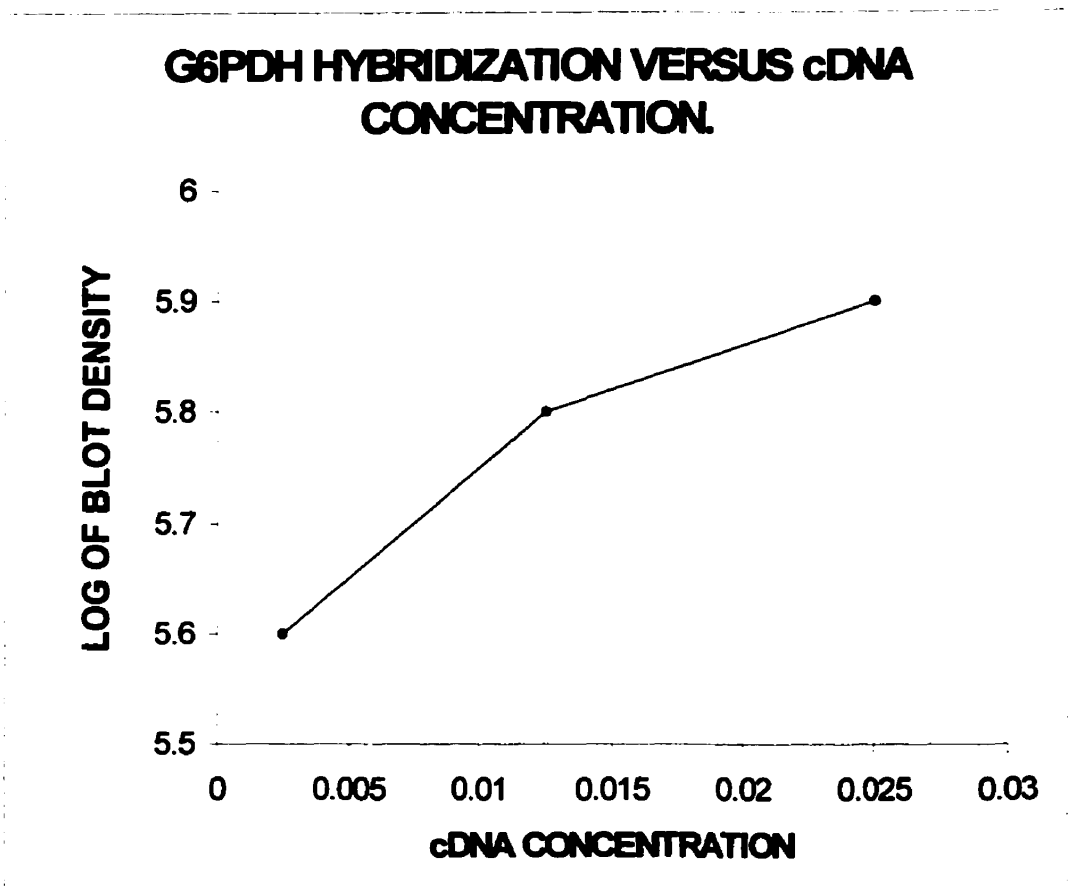

G6PDH HYBRIDIZATION VERSUS cDNA CONCENTRATION.
LOG OF BLOT DENSITY
6
5.9
5.8
5.7
5.6
5.5
0
0.005
0.01
0.015
0.02
0.025
0.03
cDNA CONCENTRATION

Figure 1. Optimization of PCR amplification of G6PDH by Titrating cDNA.

(A). γ^{32}-ATP-labelled Southern blots representing the titration of cDNA used for PCR amplification of the housekeeping gene G6PDH. In each sample assayed, cDNA was reverse transcribed from 2μg of total embryonic mRNA. The dilution values 1/40 (equivalent to 0.05μg of total RNA), 1/80 (0.025μg of total RNA) and 1/400 (0.0025μg of total RNA) signify the final concentration of reverse transcribed cDNA used in each PCR reaction. This dilution series was performed to ensure that PCR did not run to saturation levels. This assay is based on a 30-cycle amplification reaction. In addition to cDNA concentration, the $MgCl_2$ concentration was also standardized to optimize PCR for each cDNA template and primer pair. Note the decrease in band intensity as the amount of cDNA used in PCR was reduced.

(B). Graph demonstrating the log of G6PDH blot densities from fig. 1a (level of G6PDH generated by PCR) versus the concentration of cDNA initially added to the PCR reaction. Bands were visualized by Phosphorimaging and quantified with the ImageQuant program. As PCR is an exponential reaction, data was presented in a semi-log plot. Note that at a final cDNA dilution of 0.025 (equivalent to 0.05μg of total RNA), a slight deviation from linearity suggested the beginning of saturation of the PCR for G6PDH. A final dilution of 1/80, shown as 0.0125 on the graph and equivalent to 0.025μg of total RNA, provided an appropriate amount of cDNA for PCR amplification of G6PDH.

4. Detection of natural killer cell molecular markers in individual embryo implantation sites as early as 24 hours after implantation.

As it has previously been shown that NK cells can kill a wide spectrum of targets as well as secrete a number of pro-inflammatory cytokines, their presence in the decidua may lead to trophoblast cell death and early embryo demise. These experiments investigated the earliest presence of NK cells in decidual tissues. Individual embryos at day 6-8 of gestation were assayed by RT-PCR and Southern Blotting for the expression of IFNγ and perforin mRNA. In this study, IFNγ and perforin mRNA were considered to be molecular markers of decidual natural killer cells since previous immunohistochemical analysis of murine resorbing and non-resorbing decidua uncovered very few conventional CD3+ T lymphocytes (Gendron and Baines, 1988). The representative pregnancies in figures 2, 3, and 4 illustrate that as early as day 6 of gestation there was notable and differential expression of both IFNγ and perforin mRNA in nearly all the embryos analyzed in this assay. These results confirmed at the molecular level that NK cells are present in the decidua immediately following implantation and long before any overt signs of embryo demise become apparent.

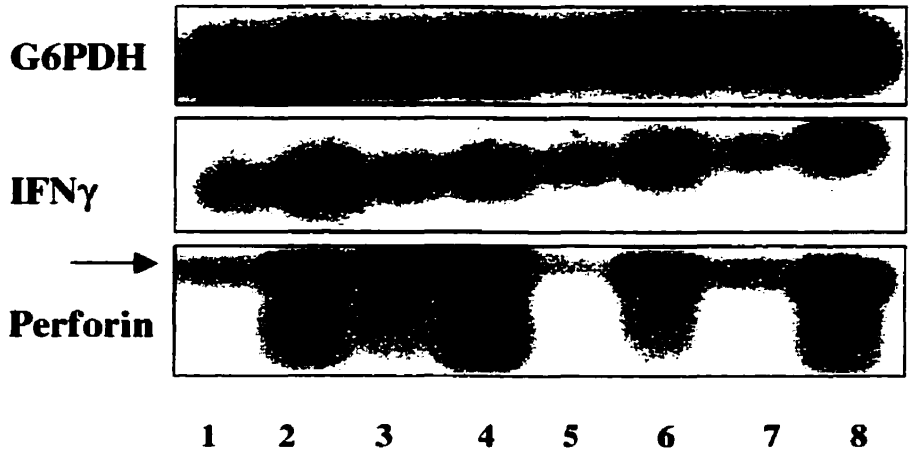
Part A of these figures display a set of representative $^{32}\gamma$-ATP-labeled Southern blots illustrating the expression of G6PDH, IFNγ, and perforin mRNA in individual (CBA/J X DBA/2) F1 embryo implants. Close examination of the radiolabeled perforin Southern blots showed the presence of two bands: a stronger and slower migrating perforin band whose amplimer size in base pairs corresponded to the expected length of 646, and a weaker faster migrating band of unknown identity. A Genebank sequence similarity search attempting to identify other genes that may be recognized by the perforin specific

primers and probe was performed and did not identify any gene other than perforin that was specific for the primer sequences used in this study. The unknown bands may be a partially degraded perforin amplimer.

The graphs in parts B and C of figures 2, 3, and 4 illustrate the densitometric analysis of IFNγ and perforin mRNA expression at days 6-8 of gestation. The levels of gene expression are represented as ratios in which the densities of IFNγ and perforin were quantified and then normalized to the respective level of expression of the housekeeping gene G6PDH in each individual embryo implant. At days 6, 7 and 8, long before any sign of overt embryonic damage and resorption were yet apparent, molecular markers of natural killer cells were detected in the decidua, suggesting that the presence of these inflammatory cytokines and the cell that produces them is not a consequence of resorption but a natural component of the implantation response. Perhaps, the presence of these inflammatory cytokines early in gestation may be due to the induction of an inflammatory response to the relatively invasive processes of implantation (day 5) and may be necessary for decidualization.

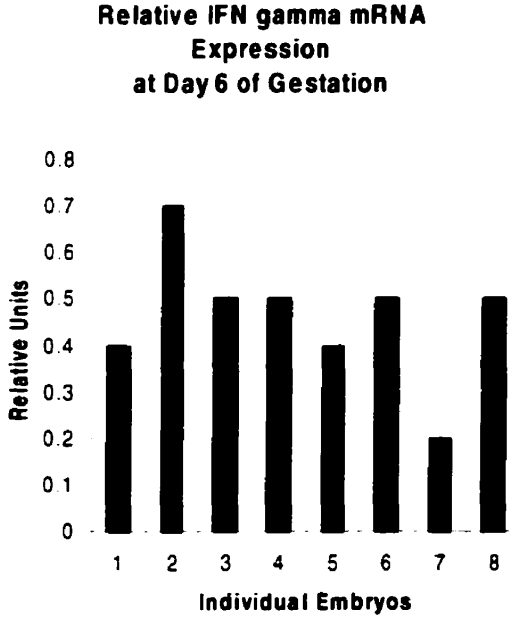
Figure 2

A.



Individual Embryos at Day 6 of Gestation

B.



C.

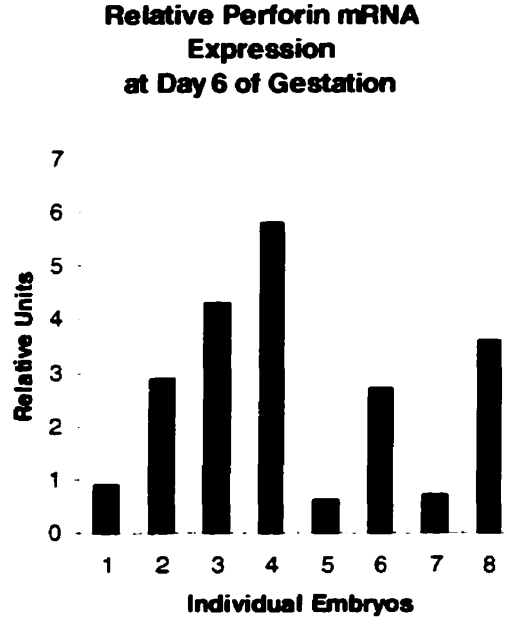
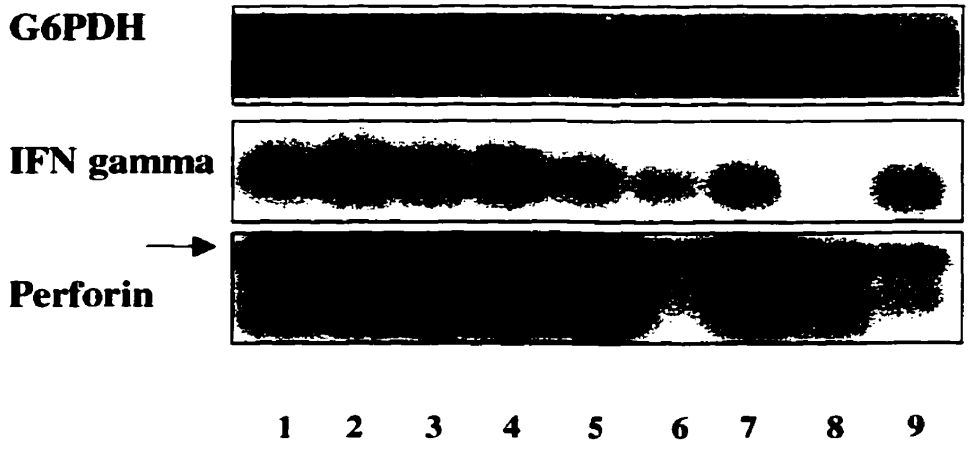


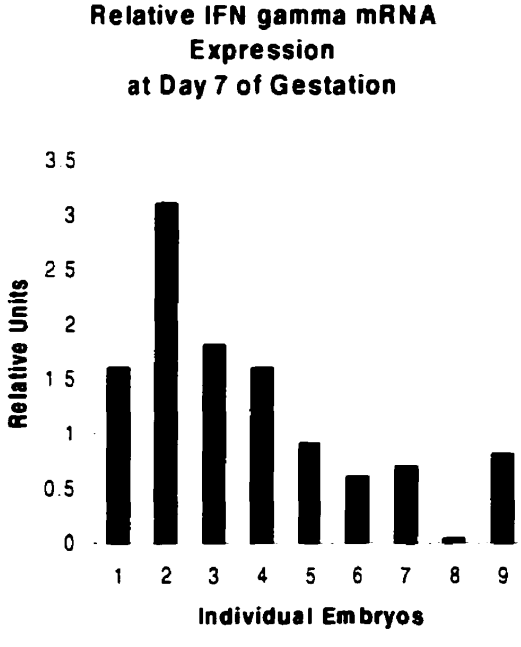
Figure 2. Representative Expression of IFNγ and Perforin mRNA in Individual Embryo Implants at *Day 6* of Gestation. Individual CBA/J X DBA/2 embryo implantation sites at day 6 of gestation were assayed by RT-PCR for the expression of IFNγ and perforin mRNA. This figure demonstrates the expression of IFNγ and perforin mRNA in one of the five pregnancies assayed at day 6. IFNγ and perforin mRNA were considered to be molecular markers of decidual NK cells. Total RNA was extracted, reverse-transcribed and PCR amplified with IFNγ and perforin specific primers. PCR products were electrophoretically separated on a 1% agarose gel and transferred onto a nylon membrane by Southern Blotting. Figure *2A* illustrates Southern Blots of IFNγ, perforin, and G6PDH PCR products hybridized with ^{32}γ-ATP-labeled IFNγ, perforin, and G6PDH oligonucleotide internal probes. Membranes were visualized by Phosphorimaging. The arrow indicates the specific perforin band. G6PDH mRNA expression was used as a control. Specific bands were quantified with the ImageQuant program. The histogram in Figure *2B* illustrates the densitometric analysis of IFNγ mRNA expression at day 6 of gestation. Figure *2C* illustrates the densitometric analysis of perforin mRNA expression at day 6 of gestation. The level of gene expression was normalized to the respective level of G6PDH expression in each embryo. Note that there was notable and differential expression of both NK cell molecular markers as early as one day following implantation.

Figure 3

A.


G6PDH
IFN gamma
Perforin
1 2 3 4 5 6 7 8 9
Individual Embryos at
Day 7 of Gestation

B.


Relative IFN gamma mRNA
Expression
at Day 7 of Gestation
Relative Units
3.5
3
2.5
2
1.5
1
0.5
0
1 2 3 4 5 6 7 8 9
Individual Embryos

C.

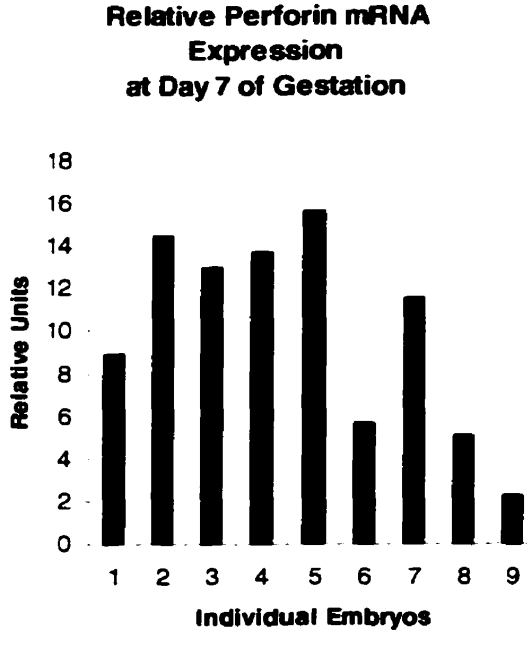
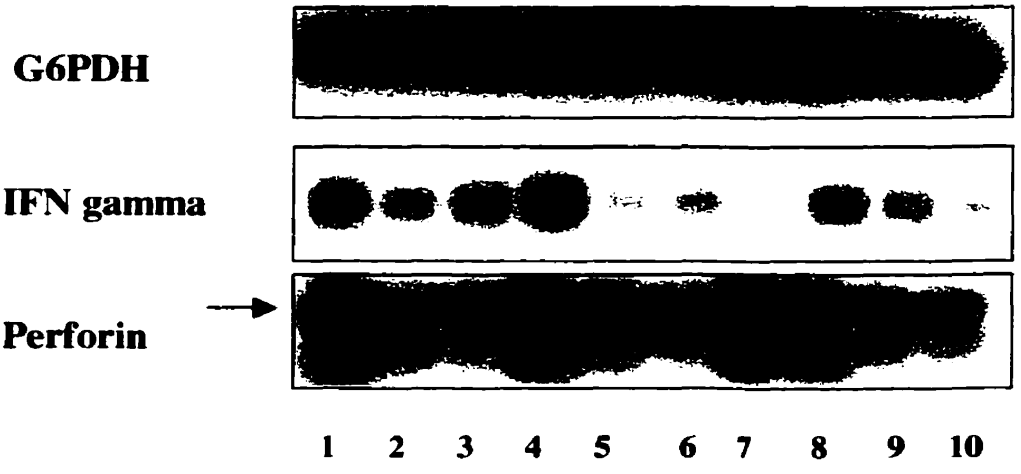

Relative Perforin mRNA
Expression
at Day 7 of Gestation
Relative Units
18
16
14
12
10
8
6
4
2
0
1 2 3 4 5 6 7 8 9
Individual Embryos

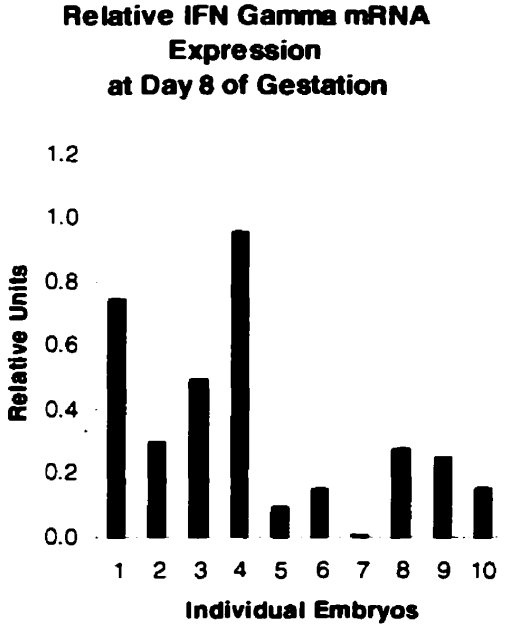
Figure 3. Representative Expression of IFNγ and Perforin mRNA in Individual Embryo Implants at *Day 7* of Gestation. Individual CBA/J X DBA/2 embryo implantation sites at day 7 of gestation were assayed by RT-PCR for the expression of IFNγ and perforin mRNA. IFNγ and perforin mRNA were considered to be molecular markers of decidual NK cells. Total RNA was extracted, reverse-transcribed and PCR amplified with IFNγ and perforin specific primers. PCR products were electrophoretically separated on a 1% agarose gel and transferred onto a nylon membrane by Southern Blotting. Figure 3*A* illustrates Southern Blots of IFNγ, perforin, and G6PDH PCR products hybridized with ^{32}γ-ATP-labeled IFNγ, perforin and G6PDH oligonucleotide internal probes. Membranes were visualized by Phosphorimaging. The arrow indicates the specific perforin band. G6PDH mRNA expression was used as a control. Specific bands were quantified with the ImageQuant program. The histogram in Figure 3*B* illustrates the densitometric analysis of IFNγ mRNA expression at day 7 of gestation. Figure *3C* illustrates the densitometric analysis of perforin mRNA expression at day 7 of gestation. The level of gene expression was normalized to the respective level of G6PDH expression in each embryo. Note the differential expression of both IFNγ and perforin mRNA among the individual embryo implantation sites. This figure demonstrates the expression of IFNγ and perforin mRNA in one of the five pregnancies assayed at day 7 of gestation.

Figure 4

A.


G6PDH
IFN gamma
Perforin
1 2 3 4 5 6 7 8 9 10
Individual Embryos at
D8 of Gestation

B.


Relative IFN Gamma mRNA
Expression
at Day 8 of Gestation
Relative Units
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1 2 3 4 5 6 7 8 9 10
Individual Embryos

C.

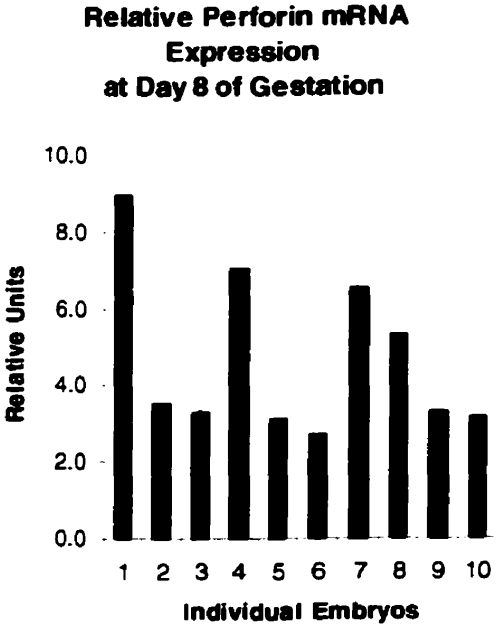

Relative Perforin mRNA
Expression
at Day 8 of Gestation
Relative Units
10.0
8.0
6.0
4.0
2.0
0.0
1 2 3 4 5 6 7 8 9 10
Individual Embryos

Figure 4. Representative Expression of IFNγ and Perforin mRNA in Individual Embryo Implants at *Day 8* of Gestation. Individual CBA/J X DBA/2 embryo implantation sites at day 8 of gestation were assayed by RT-PCR for the expression of IFNγ and perforin mRNA. IFNγ and perforin mRNA were considered to be molecular markers of decidual NK cells. Total RNA was extracted, reverse-transcribed and PCR amplified with IFNγ and perforin specific primers. PCR products were electrophoretically separated on a 1% agarose gel and transferred onto a nylon membrane by Southern Blotting. Figure 4*A* illustrates Southern Blots of IFNγ, perforin, and G6PDH PCR products hybridized with $^{32}\gamma$-ATP-labeled IFNγ, perforin, and G6PDH oligonucleotide internal probes. Membranes were visualized by Phosphorimaging. The arrow indicates the specific perforin band. G6PDH mRNA expression was used as a control. Specific bands were quantified with the ImageQuant program. The histogram in Figure 4*B* illustrates the densitometric analysis of IFNγ mRNA expression at day 8 of gestation. Figure 4*C* illustrates the densitometric analysis of perforin mRNA expression at day 8 of gestation. The level of gene expression was normalized to the respective level of G6PDH expression in each embryo. There were no obvious signs of early embryonic resorption apparent in these tissues in spite of the presence of molecular markers of natural killer cells. This figure demonstrates gene expressions in one of the five pregnancies assayed at day 8 of gestation. Note that a proportion of the embryos appeared to display increased expression IFNγ mRNA (analysis presented in figure 9, table 3 and 4).

5. Detection of macrophage activation markers in individual embryo implants at day 6, 7, and 8 of gestation.

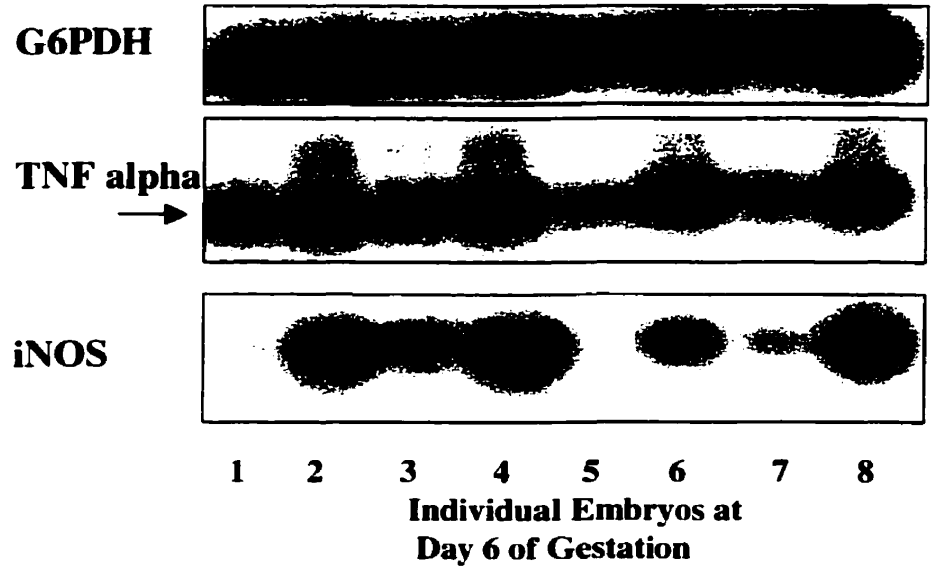
Since macrophages have previously been implicated as final inflammatory effectors of resorption at day 12 of gestation (Duclos *et al.*, 1995), and can kill a wide variety of targets through the secretion of cytotoxins (TNFα, NO), their presence in early pregnancy decidual tissue may cause early embryo resorption. In these experiments, the earliest presence of macrophages in decidual tissues was investigated. Individual CBA/J X DBA/2 F1 embryo implantation sites at day 6, 7, and 8 of gestation were assayed by RT-PCR for the expression of two macrophage activation markers, TNFα and iNOS mRNA. The representative CBA/J X DBA/2 matings shown in figures 5, 6, and 7 illustrate that at days 6-8 of gestation there was notable and differential expression of both macrophage activation markers. These results confirmed at the molecular level the decidual infiltration by activated macrophages, as early as 24 hours after implantation. Since overt embryo damage is not apparent at day 6-8 of gestation, these results further suggested that decidual infiltration by TNFα and iNOS mRNA positive cells is not a consequence of resorption. Perhaps TNFα and iNOS are natural components of the implantation response. These results are in accordance with previous immunohistochemical studies of pregnant uteri that showed the presence of a considerable number of primed macrophages in early pregnancy (Duclos *et al.*, 1995; Haddad *et al.*, 1995).

In figures 5a and 6a, Southern Blots of TNFα PCR products hybridized with radiolabeled TNFα specific internal probes revealed the presence of a very weak nonspecific band that mimics the pattern of expression of the stronger TNFα band. The

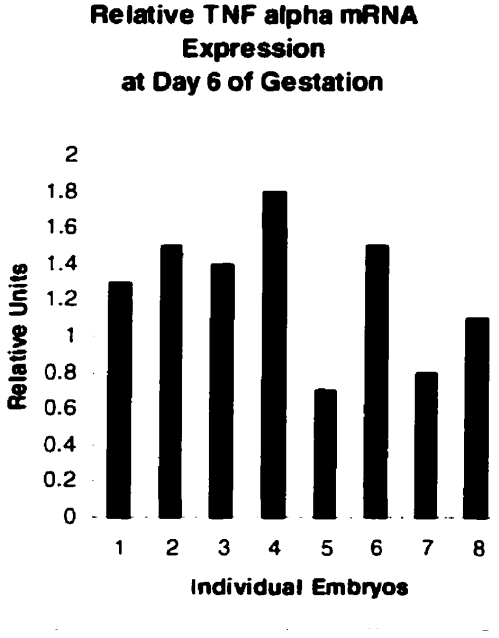
stronger band was identified as the TNFα amplimer since its size corresponds to an expected length of 498 base pairs. A Genbank sequence similarity search attempting to identify other genes that may be recognized by the TNFα specific primers and probe was performed. Although there were no murine genes other than TNFα that shared 100% sequence similarity with the chosen primers and probe, one Genbank entry referred to as Mus Musculus casein kinase 2 beta subunit did share 90% homology with the primers and 100% with the internal probe. The identity of the second weak band was not confirmed.

Figure 5

A.


G6PDH
TNF alpha
iNOS
1 2 3 4 5 6 7 8
Individual Embryos at
Day 6 of Gestation

B.


Relative TNF alpha mRNA
Expression
at Day 6 of Gestation
Relative Units
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1 2 3 4 5 6 7 8
Individual Embryos

C.

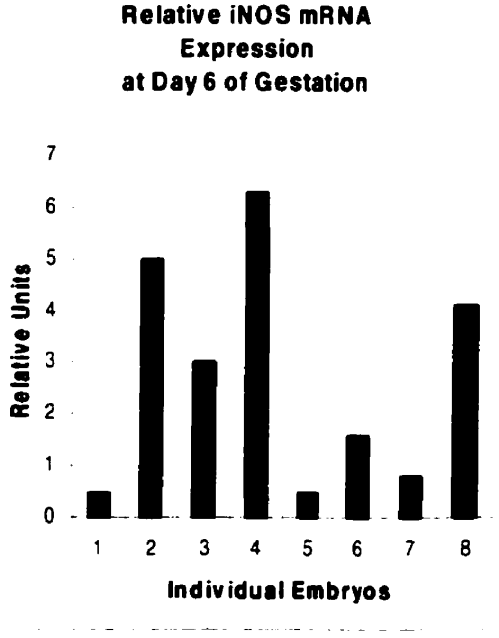
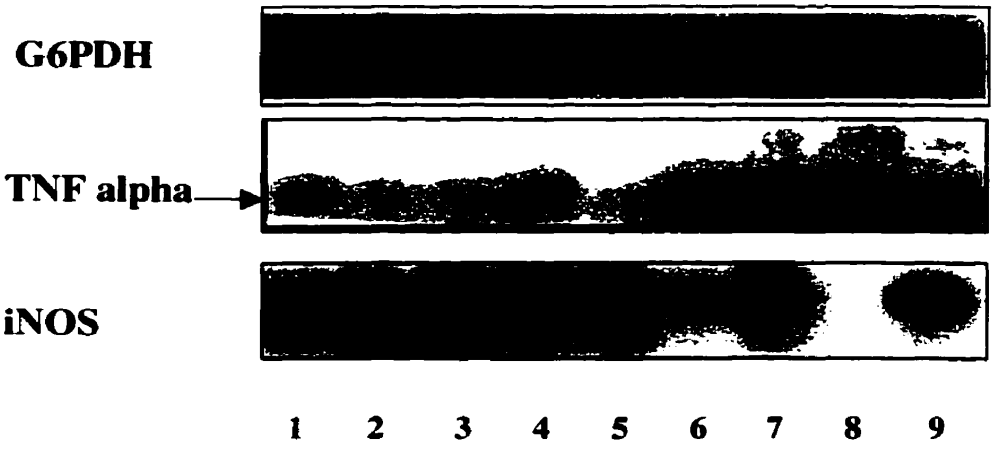

Relative iNOS mRNA
Expression
at Day 6 of Gestation
Relative Units
7
6
5
4
3
2
1
0
1 2 3 4 5 6 7 8
Individual Embryos

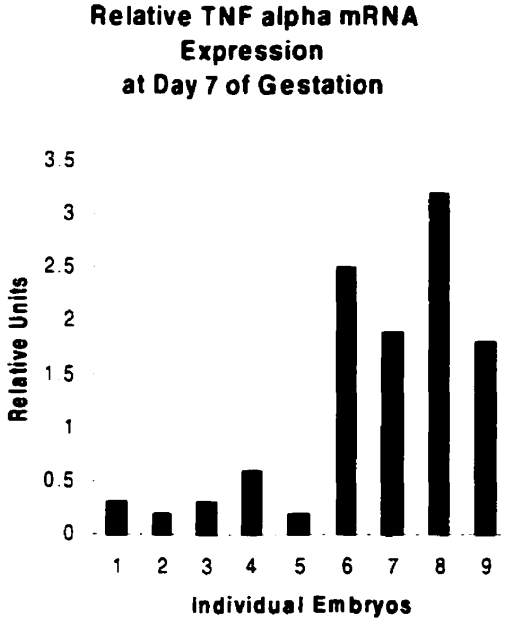
Figure 5. Representative Expression of TNFα and iNOS mRNA in Individual Embryo Implants at *Day 6* of Gestation. Individual CBA/J X DBA/2 embryo implantation sites were assayed by RT-PCR to assess the expression of TNFα and iNOS mRNA at day 6 of gestation. TNFα and iNOS mRNA served as molecular markers of macrophage activation. This figure demonstrates the expression of TNFα and iNOS mRNA in one of the five pregnancies assayed at day 6. Total RNA was extracted, reverse-transcribed, and PCR amplified with TNFα and iNOS specific primers. PCR products were electrophoretically separated on a 1% agarose gel and transferred onto a nylon membrane by Southern Blotting. Figure 5*A* illustrates Southern Blots of TNFα, iNOS, and G6PDH PCR products hybridized with $^{32}\gamma$-ATP-labeled TNFα, iNOS, and G6PDH oligonucleotide internal probes. Membranes were visualized by Phosphorimaging. The arrow indicates the specific TNFα band. G6PDH mRNA expression was used as a control. Specific bands were quantified with the ImageQuant program. The histogram in Figure 5*B* illustrates the densitometric analysis of TNFα mRNA expression at day 6 of gestation. Figure 5*C* illustrates the densitometric analysis of iNOS mRNA expression at day 6 of gestation. The level of gene expression was normalized to the respective level of G6PDH expression in each embryo. Note that the relative iNOS mRNA expression of 6.3 in embryo number 4 was an underestimation of the actual value due to a distortion of the band. There was notable and differential expression of both markers of macrophage activation as early as one day following implantation.

Figure 6

A.


G6PDH
TNF alpha
iNOS
1 2 3 4 5 6 7 8 9
Individual Embryos at
Day 7 of Gestation

B.


Relative TNF alpha mRNA
Expression
at Day 7 of Gestation
Relative Units
3.5
3
2.5
2
1.5
1
0.5
0
1 2 3 4 5 6 7 8 9
Individual Embryos

C.

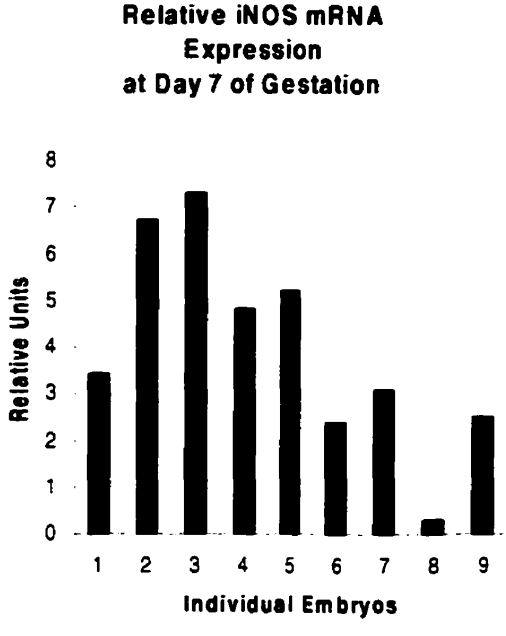
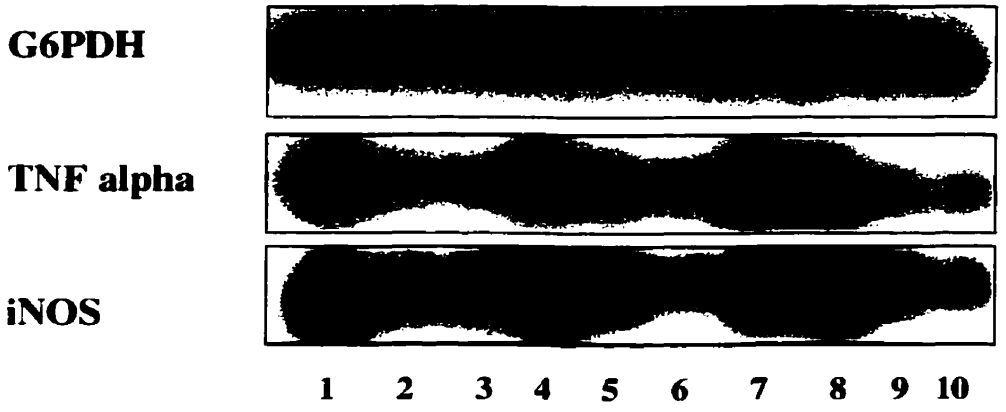

Relative iNOS mRNA
Expression
at Day 7 of Gestation
Relative Units
8
7
6
5
4
3
2
1
0
1 2 3 4 5 6 7 8 9
Individual Embryos

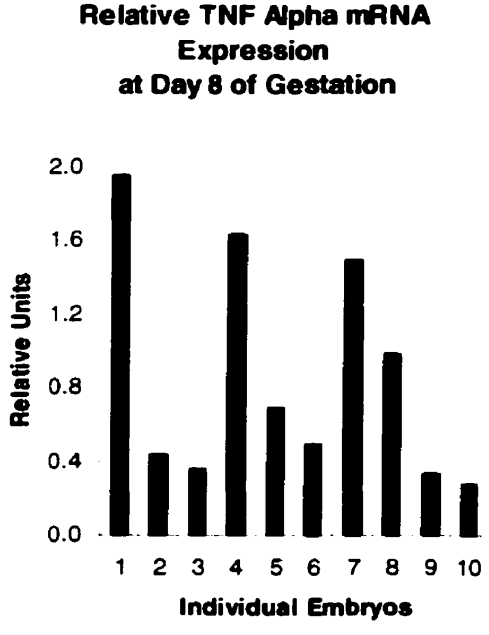
Figure 6. Representative Expression of TNFα and iNOS mRNA in Individual Embryo Implants at *Day 7* of Gestation. Individual CBA/J X DBA/2 embryo implantation sites were assayed by RT- PCR to assess the expression of TNFα and iNOS mRNA at day 7 of gestation. TNFα and iNOS mRNA served as molecular markers of macrophage activation. This figure demonstrates the expression of TNFα and iNOS mRNA in one of the five pregnancies assayed at day 7. Total RNA was extracted, reverse-transcribed, and PCR amplified with TNFα and iNOS specific primers. PCR products were electrophoretically separated on a 1% agarose gel and transferred onto a nylon membrane by Southern Blotting. Figure *5A* illustrates Southern Blots of TNFα, iNOS, and G6PDH PCR products hybridized with $^{32}\gamma$-ATP-labeled TNFα, iNOS, and G6PDH oligonucleotide internal probes. Membranes were visualized by Phosphorimaging. The arrow indicates the specific TNFα band. G6PDH mRNA expression was used as a control. Specific bands were quantified with the ImageQuant program. The histogram in Figure *6B* illustrates the densitometric analysis of TNFα mRNA expression at day 7 of gestation. Figure *6C* illustrates the densitometric analysis of iNOS mRNA expression at day 7 of gestation. The level of gene expression was normalized to the respective level of G6PDH expression in each embryo. Note that obvious signs of early embryonic resorption are not yet apparent even though molecular markers of macrophage activation are detected in individual embryo implantation sites at day 7 of gestation.

Figure 7

A.


G6PDH
TNF alpha
iNOS
1 2 3 4 5 6 7 8 9 10
Individual Embryos at
Day 8 of Gestation

B.


Relative TNF Alpha mRNA
Expression
at Day 8 of Gestation
Relative Units
2.0
1.6
1.2
0.8
0.4
0.0
1 2 3 4 5 6 7 8 9 10
Individual Embryos

C.

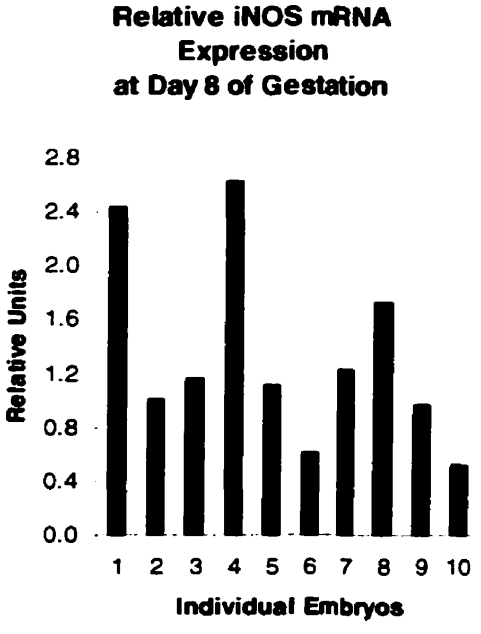

Relative iNOS mRNA
Expression
at Day 8 of Gestation
Relative Units
2.8
2.4
2.0
1.6
1.2
0.8
0.4
0.0
1 2 3 4 5 6 7 8 9 10
Individual Embryos

Figure 7. Representative Expression of TNFα and iNOS mRNA in Individual Embryo Implants at *Day 8* of Gestation. Individual CBA/J X DBA/2 embryo implantation sites were assayed by RT-PCR to assess the expression of TNFα and iNOS mRNA at day 8 of gestation. TNFα and iNOS mRNA served as molecular markers of macrophage activation. This figure demonstrates the expression of TNFα and iNOS mRNA in one of the five pregnancies assayed at day 8. Total RNA was extracted, reverse-transcribed, and PCR amplified with TNFα and iNOS specific primers. PCR products were electrophoretically separated on a 1% agarose gel and transferred onto a nylon membrane by Southern Blotting. Figure 7*A* illustrates Southern Blots of TNFα, iNOS, and G6PDH PCR products hybridized with $^{32}\gamma$-ATP-labeled TNFα, iNOS, and G6PDH oligonucleotide internal probes. G6PDH membranes were visualized and quantified by Phosphorimaging. All day 8 membranes for TNFα and iNOS were exposed to autoradiographic film and specific bands were quantified with a scanning densitometer due to a prolonged breakdown of the Phosphorimager. The histogram in Figure 7*B* illustrates the densitometric analysis of TNFα mRNA expression at day 8 of gestation. Figure 7*C* illustrates the densitometric analysis of iNOS mRNA expression at day 8 of gestation. The level of gene expression was normalized to the respective level of G6PDH expression in each embryo. Note that at day 8 of gestation a proportion of the embryos displayed increased expression of TNFα and iNOS mRNA (analysis presented in tables 3 and 4, figure 10 and 11).

*6. Natural killer cells and macrophages **selectively** infiltrate the decidua of individual implantation sites.*

In these experiments, tissue controls were analyzed by RT-PCR to compare the basal tissue activities to the events occurring at the implantation sites. The ^{32}P-hybridized Southern Blots presented in figure 8 illustrate the RT-PCR analysis of IFNγ, perforin, TNFα, and iNOS mRNA expression in 5 individual tissue controls. cDNA that was reverse-transcribed from splenic mRNA (sample number 5) served as a positive control for the expression of the housekeeping gene G6PDH as well as for IFNγ, perforin, TNFα, and iNOS. Sample numbers 1, 2, and 3 demonstrate that non-implanted portions of pregnant uterine tissue harvested from DBA/2-mated CBA/J female mice at day 6, 7, and 8 of gestation, did not express significant levels of NK cell and macrophage molecular markers. Moreover, NK cell and macrophage molecular markers were absent in non-pregnant uterine tissue as demonstrated by sample number 4. Collectively, since significant levels of NK cell and macrophage molecular markers are not detected in non-implanted uterine tissues, it can be inferred that during normal pregnancy, maternal inflammatory cells selectively infiltrate the decidua of embryo implants as early day 6 of gestation.

Figure 8

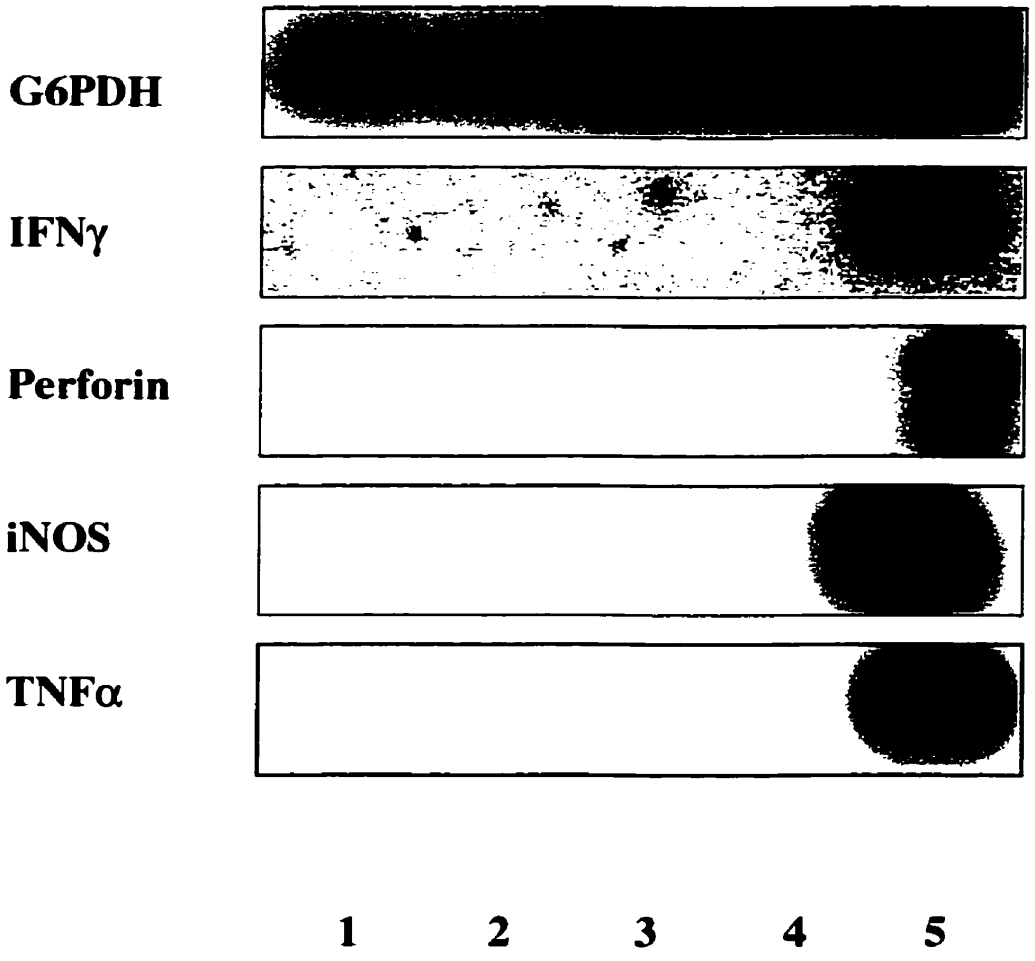


Figure 8. RT-PCR Analysis of Macrophage and NK Cell Molecular Markers in Tissue Controls. Non-implanted portions of pregnant CBA/J uteri at day 6-8, non-pregnant uteri and spleen from CBA/J mice were assayed by RT- PCR to assess the expression of TNFα, iNOS, perforin, and IFNγ mRNA. TNFα and iNOS mRNA served as molecular markers of macrophage activation while perforin and IFNγ were considered to be molecular markers of decidual NK cells. For technical details see legend for figure 2. $^{32}\gamma$-ATP-labeled Southern Blots of IFNγ, perforin, TNFα, and iNOS PCR products were visualized with a Phosphorimager. The constitutively activated housekeeping gene G6PDH was assayed to assess the relative quantity of the extracted mRNA in each tissue sample. Note that molecular analysis did not detect significant expression of NK cell and macrophage specific molecular markers in non-implanted uteri and non-pregnant uteri.

Sample 1. Non-implanted portion of pregnant CBA/J uterus at day 6.

Sample 2. Non-implanted portion of pregnant CBA/J uterus at day 7.

Sample 3. Non-implanted portion of pregnant CBA/J uterus at day 8.

Sample 4. Non-pregnant CBA/J uterus.

Sample 5. Spleen from CBA/J female mice as a positive control.

7. Analysis of perforin, IFNγ, TNFα, and iNOS mRNA expression at day 8 of gestation.

Previously, immunohistochemistry has demonstrated that the density of macrophage and natural killer cell infiltration displays a distinct bimodal distribution among CBA/J X DBA/2 F1 implantation sites at day 8 of gestation (Duclos *et al.*, 1995; Gendron and Baines, 1988). Recent studies have also suggested that the expression of iNOS and TNFα mRNA at day 8 of gestation show a bimodal distribution (Haddad *et al.*, 1997). In both of these studies, CBA/J X BALB/c (low loss) mating pairs were used to determine the cell number or relative expression unit that classified CBA/J X DBA/2 (high loss) embryos as normal or resorbing. In the present experiments, a proportion of CBA/J X DBA/2 F1 embryos appeared to express increased levels of TNFα, iNOS, perforin and IFNγ mRNA at day 8 of gestation (figures 4 and 7). To verify this hypothesis, histograms depicting the frequency distribution of both natural killer cell and macrophage markers at day 8 of gestation were plotted as illustrated in figures 9 and 10. Table 3 summarizes the statistics employed to analyze the normality of the frequency distribution histograms. IFNγ, TNFα, and iNOS mRNA expression at day 8 appeared to show an asymmetrical skewed distribution to the right indicating a bimodal type of distribution. Conversely, perforin mRNA expression appeared to display a normal distribution among the 43 embryos analyzed.

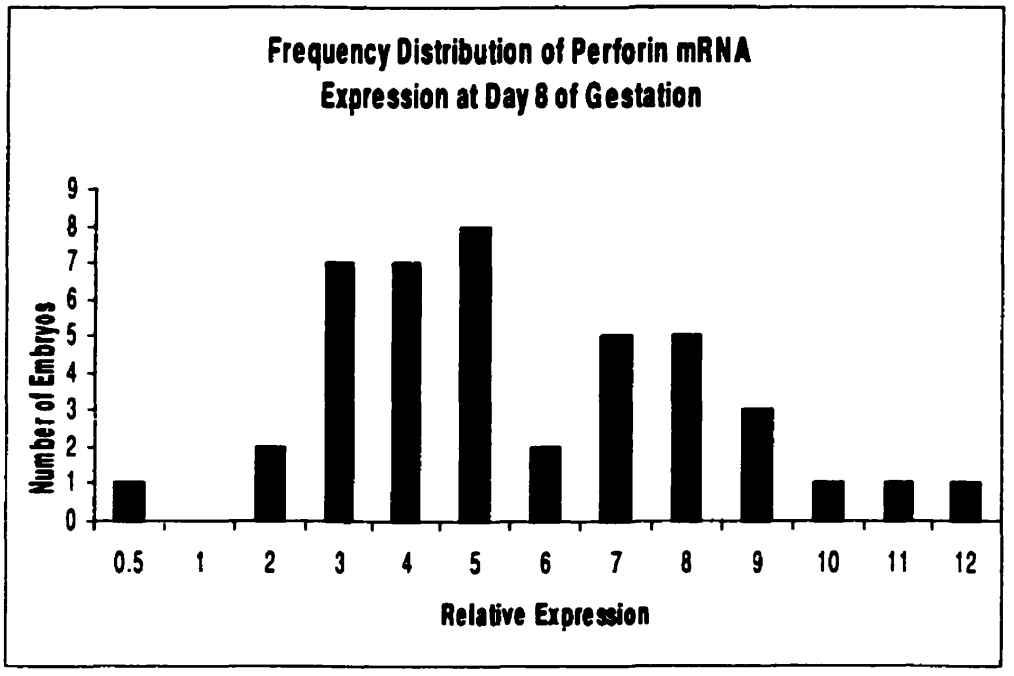
Where bimodal distributions were apparent (IFNγ, TNFα, and iNOS), K-means cluster analysis was employed to determine the approximate mean of the embryonic population that exhibited the lower and presumably normal level of gene expression (table 4). Using these K-means, an upper 95% confidence limit (UCL95%) of 0.5 was determined for IFNγ, 0.7 for TNFα, and 1.2 for iNOS. Day 8 CBA/J X DBA/2 embryos with gene expression levels less than the UCL95% were considered to be normal and

non-resorbing. Embryos with gene expression levels greater than the UCL95% were considered as potentially resorbing, since expression of resorption-associated cytokines was significantly increased. Using the UCL95% as a reference point, it was determined that 26% of embryos exhibited significantly increased expression of TNFα mRNA and 30% exhibited significantly increased levels of iNOS mRNA expression. These results are in accordance with previous studies and these percentages correspond to the rate of early embryo loss observed in this high loss mating model (20-30%). However, the proportion of embryos displaying significantly increased IFNγ mRNA expression (44%) was higher than the 20-30% resorption rate observed in this high loss mating pair. Therefore, at day 8 of gestation, before any signs of resorption had become obvious, a proportion of embryos expressed significantly increased levels of TNFα, iNOS, and IFNγ mRNA. These results agree with previous studies that showed an association between molecular markers of macrophage activation and early embryo loss (Haddad *et al.*, 1997).

Perforin mRNA expression displayed a normal distribution and appeared constitutively high in most of the embryos assayed. As seen in figure 9A, there was a wide range of perforin mRNA expression values (0.5 to 12). The distribution appeared symmetrical as the mean coincided with the median and the null hypothesis could not be rejected, as the ratio of skewness to its standard error was determined as 1 (table 3). Given that NK cells can exert direct cytotoxicity by releasing the pore-forming protein perforin, these results agree with the hypothesis that NK cells mediate early embryo loss indirectly, perhaps by releasing pro-inflammatory cytokines that activate decidual macrophages.

Figure 9

A.


Frequency Distribution of Perforin mRNA Expression at Day 8 of Gestation
Number of Embryos
0
1
2
3
4
5
6
7
8
9
0.5
1
2
3
4
5
6
7
8
9
10
11
12
Relative Expression

B.

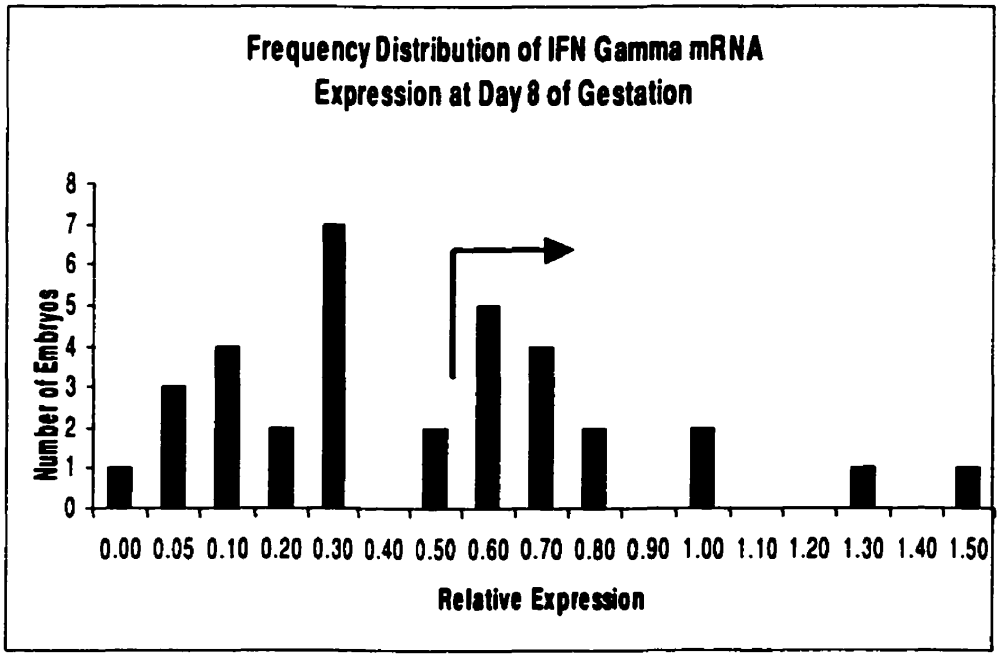
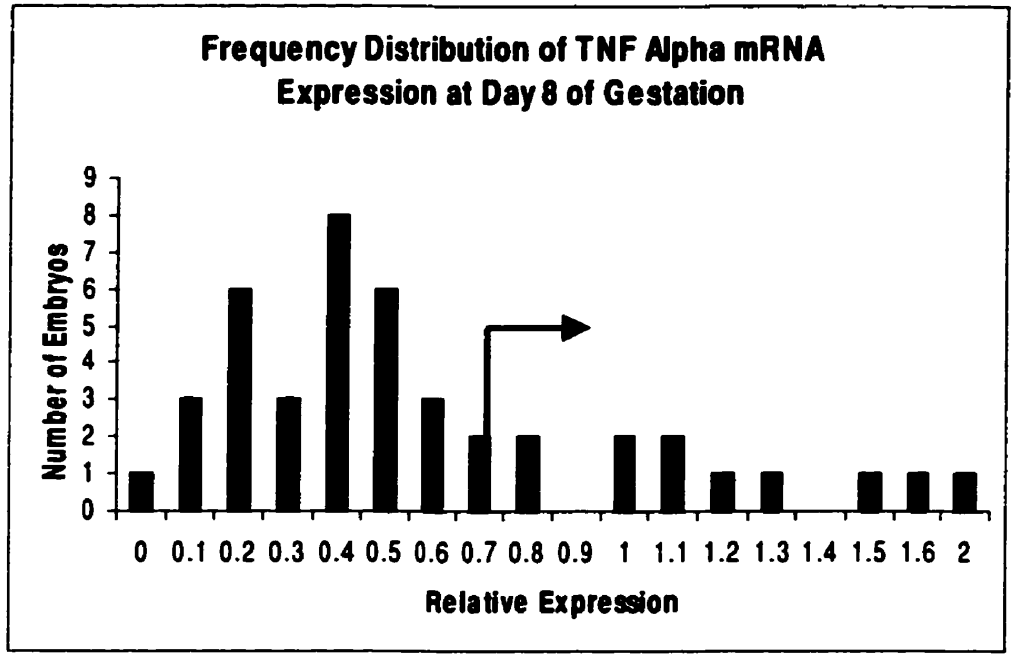

Frequency Distribution of IFN Gamma mRNA Expression at Day 8 of Gestation
Number of Embryos
0
1
2
3
4
5
6
7
8
0.00
0.05
0.10
0.20
0.30
0.40
0.50
0.60
0.70
0.80
0.90
1.00
1.10
1.20
1.30
1.40
1.50
Relative Expression

Figure 9. Frequency Distribution of A) Perforin and B) IFNγ mRNA Expression at Day 8 of Gestation. Frequency distribution histograms were plotted to verify the hypothesis that at day 8 a proportion of CBA/J X DBA/2 F1 embryo implantation sites expressed significantly increased levels of perforin and IFNγ mRNA. Table 3 summarizes the statistics employed to analyze the normality of the frequency distribution histograms. Where bimodal distributions were apparent, K means cluster analysis was employed to determine the mean and 95% UCL of the embryonic population exhibiting lower and presumably normal level of gene expression (table 4). For technical details of RT-PCR amplification see legend of figure 4. Southern Blots of Perforin, IFNγ and G6PDH PCR products were hybridized with radiolabeled gene specific internal probes, visualized by phosphorimaging and quantified with the ImageQuant program. The level of gene expression was normalized to the respective level of G6PDH expression in each embryo implantation site. Perforin mRNA expression appeared to display a broad but normal distribution ranging from 0.5 to 12. The null hypothesis was not rejected as the ratio of skewness to its standard error was determined as 1. On the other hand, IFNγ mRNA expression appeared to be skewed to the right. The arrow in *9B* refers to the 95%UCL (relative expression = 0.5). Expression of IFNγ mRNA greater than 0.5 was categorized as significantly increased. 44% of the embryos had significantly increased IFNγ mRNA expression. The relative gene expression per embryo implantation sites is indicated on the abscissa, ranging by 1 in figure *9A* (eg. 5 represents the range from 4.1 to 5), and by 0.1 in figure *9B* (eg. 0.5 represents the range from 0.41 to 0.5).

Figure 10

A.


Frequency Distribution of TNF Alpha mRNA Expression at Day 8 of Gestation
Number of Embryos
0 1 2 3 4 5 6 7 8 9
0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1 1.1 1.2 1.3 1.4 1.5 1.6 2
Relative Expression

B.

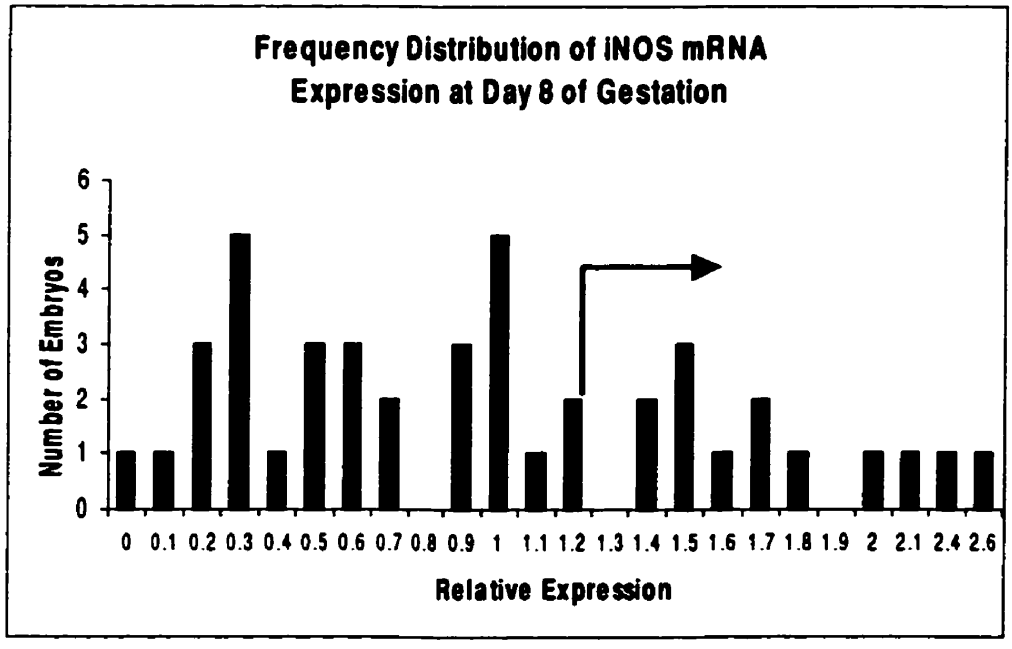

Frequency Distribution of iNOS mRNA Expression at Day 8 of Gestation
Number of Embryos
0 1 2 3 4 5 6
0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1 1.1 1.2 1.3 1.4 1.5 1.6 1.7 1.8 1.9 2 2.1 2.4 2.6
Relative Expression

Figure 10. Frequency Distribution of A) TNFα and B) iNOS mRNA Expression at Day 8 of Gestation. Frequency distribution histograms were plotted to verify the hypothesis that at day 8 a proportion of CBA/J X DBA/2 F1 embryo implantation sites expressed significantly increased levels of TNFα and iNOS mRNA. Table 3 summarizes the statistics employed to analyze the normality of the frequency distribution histograms. Where bimodal distributions were apparent, K means cluster analysis was employed to determine the mean and 95% UCL of the embryonic population exhibiting lower and presumably normal level of gene expression (table 4). For technical details of RT-PCR amplification and Southern Blotting see legend of figure 7. The level of gene expression was normalized to the respective level of G6PDH expression in each embryo implantation site. Both TNFα and iNOS mRNA expression appear to be skewed to the right. The arrows refers to the 95%UCL (0.7 for TNFα, 1.2 for iNOS). Expression of TNFα mRNA greater than 0.7 was categorized as significantly increased. Expression of iNOS mRNA greater than 1.2 was categorized as significantly increased. 26% of the embryos had significantly increased TNFα mRNA expression and 30% had significantly increased levels of iNOS mRNA. The relative gene expression per embryo implantation sites is indicated on the abscissa, ranging by 0.1 both in figure *10A* and figure *10B (*eg. 0.5 represents the range from 0.41 to 0.5).

Table 3. Statistical Analysis of Day 8 Frequency Distributions of Relative Perforin, IFNγ, TNFα and iNOS mRNA Expression.

CYTOKINE	NUMBER OF EMBRYOS	MEAN [a] EXPRESSION	MEDIAN [b] EXPRESSION	SKEWNESS STATISTIC/ STD. ERROR RATIO [c]
PERFORIN	43	5.70	5.10	1
IFNγ	34	0.47	0.41	2
TNFα	43	0.58	0.45	4
INOS	43	0.96	0.92	2

a,b: A comparison of the means and medians of relative gene expression were used as measures of central tendency. When the mean was greater than the median, the data distribution was skewed to the right.

c: Skewness measures the asymmetry of a sample distribution. In this study, normality was rejected if the ratio of skewness to its standard error was determined as ≥ 2.

Table 4. K Means Cluster Analysis at Day 8 of Gestation

CYTOKINE	NUMBER OF EMBRYOS	MEAN OF CLUSTER 1*	UPPER 95% CONFIDENCE LIMIT	% OF EMBRYOS WITH INCREASED EXPRESSION
IFNγ	34	0.1968	0.5	44
TNFα	43	0.3862	0.7	26
INOS	43	0.5834	1.2	30

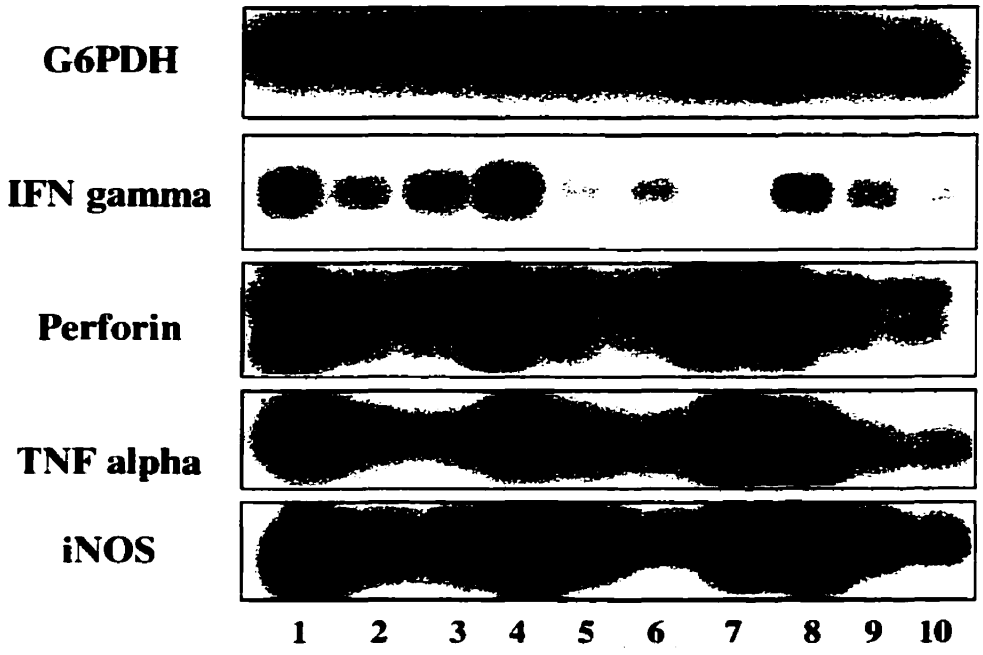
*Note. Where bimodal distributions were apparent, "mean of cluster 1" or the mean and standard deviation of the population of embryos with the lower relative gene expression were used to determine the 95% UCL.

8. Simultaneous increased expression of TH1 inflammatory cytokines.

In these experiments it was also determined that at day 8 of gestation, a portion of embryos showed simultaneous increased expression of both NK and macrophage-associated molecular markers. The Southern Blots in figure 11 depict one of the 5 CBA/J X DBA/2 matings analyzed at day 8 of gestation. As presented in the table of figure 11, embryo number 1, 4, 7 and 8 expressed significantly increased levels of macrophage activation markers TNFα and iNOS mRNA. Along with increased TNFα and iNOS mRNA expression, embryos number 1 and 4 (20%) also expressed significantly elevated levels of IFNγ mRNA. Interestingly, TNFα and iNOS mRNA expression was increased to a greater extent in the embryos also expressing increased IFNγ mRNA (embryo 1 and 4). Perforin mRNA expression displayed a normal distribution and appeared to be constitutively high in all the embryos. The percentage of embryos with increased expression of IFNγ, TNFα and iNOS mRNA (20%) corresponded to the rate of early embryo loss in this mating model (20-30%). Since TNFα and iNOS are cytotoxic effector factors produced by activated macrophages, and IFNγ is a major macrophage activator, these results confirmed previous findings that early embryo loss is associated with increased decidual infiltration by activated macrophages (Haddad *et al.*, 1997). Furthermore, these molecular results suggest that early embryo loss is not caused by perforin mediated NK cytotoxicity since the level of perforin mRNA expression was not substantially increased in potentially resorbing embryos. Finally, there were instances when the embryos expressed increased levels of one cytokine and not of the other.

Figure 11

A.



B.

EMBRYO	PERFORIN	IFN GAMMA	TNF ALPHA	iNOS
1	+	+	+ +	+ +
2	+	-	-	-
3	+	-	-	-
4	+	+ +	+ +	+ +
5	+	-	-	-
6	+	-	-	-
7	+	-	+	+
8	+	-	+	+
9	+	-	-	-
10	+	-	-	-

Figure 11. Simultaneous Increased Expression of IFNγ, Perforin, TNFα, and iNOS mRNA. Individual CBA/J X DBA/2 embryo implantation sites were assayed by RT-PCR to assess the simultaneous expression of macrophage and NK cell molecular markers at day 8 of gestation. This figure demonstrates the cytokine mRNA expression in one of the five pregnancies assayed at day 8. For technical details see legend for figure 4 and 7. G6PDH, perforin, and IFNγ blots were visualized by Phosphorimaging and quantified with the ImageQuant program. TNFα and iNOS blots were exposed to autoradiographic film and quantified with a scanning densitometer due to the prolonged breakdown of the Phosphorimager. Figure *11A* shows Southern Blots of IFNγ, Perforin, TNFα, iNOS, and G6PDH PCR products hybridized with ^{32}γ-ATP-labeled cytokine specific internal probes. Respective G6PDH densities were used to normalize IFNγ, perforin, TNFα, and iNOS mRNA expression levels. Figure *11B* depicts those embryos that displayed elevated cytokine mRNA expression (i.e. mRNA expression greater than the 95% UCL) with a + sign. ++ is assigned to embryos expressing mRNA levels at least 1X greater than the 95%UCL. A – sign is ascribed to non-resorbing embryos that expressed normal levels of TH1 cytokines (i.e. less than the 95%UCL). As perforin mRNA expression displayed a normal distribution among the embryos, they were categorized with a + sign. Note that embryo 1 and 4 had elevated expression of all 4 cytokines. Embryo 7 and 8 displayed elevated expression of TNFα and iNOS mRNA but to a lesser extent than embryos 1 and 4.

9 Identification of the source of decidual and splenic IFNγ mRNA.

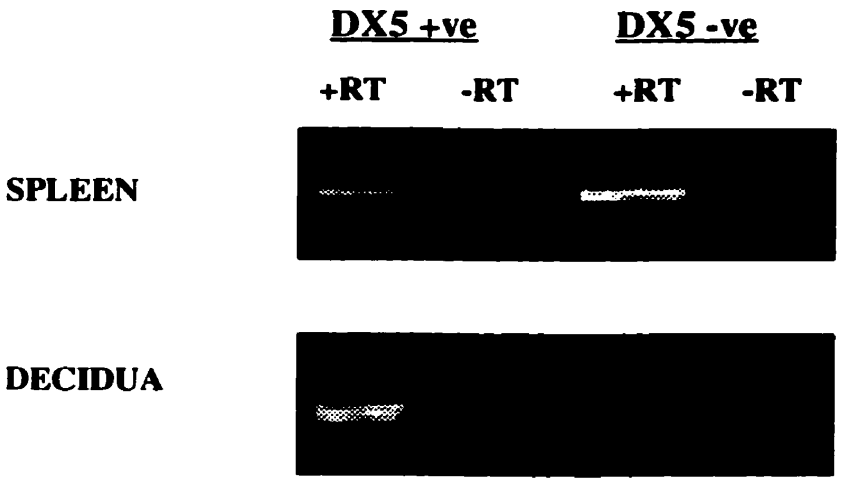
If the role of decidual NK cells is not mediating a direct cytotoxic attack on embryonic trophoblast cells, they may be responsible for producing cytokines that activate decidual macrophages. IFNγ is a major activator of macrophages, can provoke fetal demise (Chaouat *et al.*, 1990) and is associated with early embryo loss (Haddad *et al.*, 1997a). In these preliminary experiments, cell labeling with an anti-NK antibody (DX5), cell sorting, and RT-PCR gene expression analysis were performed to confirm the source of IFNγ mRNA in the decidua and spleen of DBA/2-mated CBA/J females (table 5). As shown in the agarose gel of figure 12, splenic DX5+ve and DX5-ve cells expressed IFNγ mRNA, decidual DX5+ve cells expressed IFNγ whereas decidual DX5 negative cells did not appear to express IFNγ mRNA. These preliminary results suggest that IFNγ was produced by more than one type of cell in the spleen, while decidual IFNγ mRNA was produced solely by DX5+ve cells. Since DX5 is an NK cell specific marker, IFNγ mRNA expression at the fetal-maternal interface may be an appropriate molecular marker for studying natural killer cell involvement in early embryo loss.

Table 5. FACS/SORT of Murine Spleen and Decidual Cells Expressing DX5 at Day 9 of Gestation.

Source of Cells	% of $DX5^+$ Cells Prior to Sorting	Number of Sorted Cells*	
		$DX5^+$	$DX5^-$
Spleen	3.26	9×10^4	3×10^5
Embryos	1.86	1.24×10^5	5×10^5

*Note. Refers to the number of recovered cells from which RNA was extracted to perform RT-PCR analysis of IFNγ mRNA expression.

Figure 12



Agarose Gel of RT-PCR Analysis for IFNγ mRNA Expression in DX5 +ve and DX5 -ve Cells.

Figure 12. Agarose Gel of RT-PCR Analysis of IFNγ mRNA Expression in DX5+ve and DX5-ve cells. CBA/J X DBA/2 F1 embryo implants and spleen were assayed by RT-PCR to investigate the source of IFNγ mRNA in these tissues. Uterine and spleen were removed from pregnant CBA/J mice at day 9 of gestation and the individual embryo implants were pooled together to increase the cell yield. Cell suspensions were prepared in Dulbecco's PBS + 2% fetal calf serum before staining. PE-conjugated rat anti-mouse pan NK cell monoclonal antibody DX5 was used to label decidual and spleen cells that express the natural killer cell surface marker. Fc Block, a rat anti-mouse CD16/CD32 monoclonal antibody was added to block nonspecific binding of phycoerythrin to FcγII receptors on B lymphocytes. The reaction was incubated in the dark for 15 minutes, washed and analyzed by flow cytometry. Following cell sorting to separate the DX5 negative cells from the positively stained cells, RT-PCR was performed to detect IFNγ mRNA. Total RNA was extracted, DNAse treated, reverse-transcribed, and PCR amplified with IFNγ specific primers. This assay was based on a 45-cycle amplification reaction. The PCR products were electrophoretically separated in a 1% agarose gel that contained ethidium bromide and were visualized by UV light and photo-documented. –ve RT refers to those samples in which no reverse transcriptase was added to RT-PCR. The IFNγ band corresponds to an expected length of 426 base pairs. Note that IFNγ mRNA was detected in both splenic DX5+ve and DX5-ve cells whereas only DX5+ve cells expressed IFNγ mRNA in the decidua.

Discussion

Spontaneous early embryo loss is a common complication of mammalian reproduction. The CBA/J X DBA/2 murine model has greatly facilitated the investigation of the mechanisms that cause early embryo loss. Studies on this model have indicated that embryo resorption is recurrent, partner specific (Chaouat *et al.*, 1988) and unrelated to paternal MHC expression. Furthermore, numerous findings have associated early embryo resorption with activation of decidual NK cells and macrophages, and production of TH1 inflammatory cytokines (Gendron *et al.*, 1988; Duclos *et al.*, 1995; Haddad *et al.*, 1997b). In this study, gene expression analyses were undertaken to investigate the earliest events in the activation of decidual NK cells and macrophages in initiating early embryo loss with the objective of determining when the primary causative factors are active. Very early expression could indicate a genetic or endogenous event, whereas later activation of NK cells and macrophages may indicate an exogenous pathogen-driven process. The results support and reinforce a conceptual model (Baines *et al.*, 1997) proposing that early decidual NK cell production of IFNγ leads to macrophage activation and early embryonic demise in DBA/2-mated CBA/J female mice, implicating a genetic factor.

IF NK cells and macrophages predispose embryos to early loss, it would be reasonable to expect their presence in the deciduum before embryo pathology ensues. In this study, the earliest presence of NK cells and macrophages in decidual tissues was investigated. The data demonstrated that as early as day 6 of gestation, long before obvious signs of early embryo resorption are apparent, NK cell (IFNγ and perforin

mRNA) and macrophage (TNFα and iNOS mRNA) activation markers are detected in the deciduum. Demonstrating that decidual infiltration by these inflammatory cells is not a consequence of resorption, these results also suggested that the maternal innate response has a role in implantation within 24 hours following blastocyst attachment to the uterine wall. Implantation is an invasive process that leads to the decidualization of the endometrium and the intimate juxtaposition of maternal and fetal tissues. Detection of significant levels of IFNγ, perforin, TNFα, and iNOS mRNA in nearly all placentae at day 6 of gestation, but not in non-implanted uterine tissues, inferred that NK cells and macrophages selectively infiltrate the deciduum as early as one day following implantation. These findings implied that NK cells and macrophages may be natural components of the implantation response and are consistent with previous investigations of early post-implantation events in other murine mating pairs (Hunt, 1994; Hunt and Robertson, 1996; Whitelaw and Croy, 1996; Platt and Hunt, 1998). Further, these data confirmed at the molecular level, previous studies that revealed the presence of asialo GM1-expressing NK cells and F4/80-expressing macrophages in all CBA/J X DBA/2 F1 implantation sites at day 8 of gestation (Gendron and Baines, 1988; Duclos *et al.*, 1995). The data was also consistent with previous studies that have demonstrated a significantly decreased resorption rate in DBA/2-mated CBA/J mice following early treatment with agents that abrogate NK cell and macrophage activities (deFougerolles and Baines, 1987; Duclos *et al.*, 1994).

During pregnancy, allogeneic trophoblast cells come into direct contact with the maternal immune system. It has been suggested that this maternal immunostimulation induces the production of cytokines that promote decidualization of the endometrium and

appropriate vascularization of the placenta and nutrition of the developing conceptus (Beaman, 1990). In this study, detection of notable levels of IFNγ, perforin, TNFα, and iNOS mRNA in nearly all the embryos assayed at day 6-8 of gestation is in accord with this hypothesis as well as with recent reports that have implicated pro-inflammatory cytokines in the normal processes of reproduction and pregnancy. The early presence of IFNγ and IFNγ receptors in decidualized uteri has been postulated to influence uterine hematopoietic cell development and decidualization (Platt and Hunt, 1998; Chen *et al.*, 1994). Uterine leukocyte production of nitric oxide has been proposed to promote smooth muscle relaxation and healthy vascularization of the implantation sites (Hunt *et al.*, 1997b). The early presence of TNFα has been demonstrated in normal pregnancies and has been shown to be important for normal placental growth and function (Hunt, 1996; Gorivodsky *et al.*, 1998), and even normal embryonic development (Gendron *et al.*, 1991). Finally, as the present study did not differentiate between the gene expression of the embryo and the gene expression of the placenta, a contribution by the developing conceptus itself could not be discounted, even though at day 6 of gestation such contribution should be minimal.

Another objective of this study was to investigate and detect the earliest expression of IFNγ, perforin, TNFα and iNOS mRNA as a correlate with early embryo loss. Although lower levels of pro-inflammatory cytokines might be favorable to pregnancy, abnormally high production might be detrimental to fetal survival. Indeed, injection of gravid CBA/J mice with recombinant IFNγ and TNFα has been shown to raise the rate of resorption (Chaouat *et al.*, 1990). Pooled placentae from resorption-prone mating combinations have been shown to contain significantly higher levels of

TH1 inflammatory cytokines than placentae from low loss mating pairs (Tangri and Raghupathy, 1993). Although these experiments associated TH1 cytokines with embryo death, they did not take into account the fact that resorbing and non-resorbing embryos from the same high loss pregnancy might display distinctly different TH1 cytokine expression patterns. In the present study, the early expression of IFNγ, TNFα, perforin, and iNOS mRNA was investigated at the single embryo level.

The present study detected the earliest differential expression of macrophage and NK cell activation markers as a correlate with future early embryo loss, at day 8 of gestation. Specifically, the results at day 8 of gestation revealed two major populations of embryos in regard to their expression of IFNγ, TNFα, and iNOS mRNA: one population displaying lower levels of cytokine expression, and another population exhibiting substantially increased levels of cytokine gene expression. 26%, 30%, and 44% of embryos expressed substantially elevated levels of TNFα, iNOS, and IFNγ mRNA expression, respectively. Given that the percentage of embryos with substantially increased expression of TNFα (26%) and iNOS (30%) mRNA corresponded to the rate of early embryo loss (20-30%), these results reaffirmed a role for macrophage activation in the mechanism that precedes embryo demise. Further, the increased expression of IFNγ mRNA in 44% of the fetoplacental units at day 8 of gestation, albeit greater than the incidence of early embryo loss, corroborated with earlier studies that have indicated the presence of primed macrophages in early pregnancy decidual tissues (Haddad *et al.*, 1995; Gendron *et al.*, 1990).

It is well established that macrophage activation is a multi-step process that requires the action of a number of factors. IFNγ is the best-described macrophage-

activating factor (MAF) (Ding *et al.*, 1988) and has been associated with early embryo loss (Haddad et al., 1997a). Since IFNγ can act synergistically with TNFα or bacterial lipopolysaccharide (LPS) to induce macrophage elimination of intracellular parasites (Munoz-Fernandez *et al.*, 1992), it has been hypothesized that early embryo loss results from a similar mechanism (Baines *et al.*, 1997). Given that treatment of gravid CBA/J female mice with rat anti-mouse IFNγ anti-sera rescues embryos fated to resorb at day 12 (Haddad et al., 1997a), it has been hypothesized that IFNγ primes or activates decidual macrophages and that TNFα or LPS subsequently triggers the effector functions (TNF and NO). By detecting increased levels of macrophage activation markers in a proportion of embryos that corresponded to the incidence of early embryo loss, this study supported the above-mentioned hypothesis. Detection of increased levels of IFNγ and macrophage priming was also consistent with previous studies which showed that early treatment of gravid CBA/J mice with LPS induces increased production of uterine TNFα and resorption of nearly all embryos by day 12 of gestation (Gendron *et al.*, 1990). Yet, as NK cells have also been shown to produce TNFα upon activation (Peters *et al.*, 1986; Hill, 1992; Haddad *et al.*, 1997b), questions have arisen pertaining to the use of TNFα as a molecular marker of macrophage activation. Recent demonstration that antibody-induced reduction of macrophage infiltration resulted in a significant reduction in the number of CBA/J X DBA/2 F1 embryos with increased levels of TNFα mRNA, has implicated macrophages as the primary producers of decidual TNFα (G. Youseph, personal communication). However, it cannot be discounted that NK cell production of TNFα might act as the macrophage-triggering agent.

Perforin is a pore-forming cytolysin that is stored in cytoplasmic granules and secreted by killer lymphocytes (Liu *et al.*, 1995). To demonstrate the early involvement of NK cells in early embryo loss, this study employed RT-PCR to detect the presence perforin and IFNγ mRNA. The data demonstrated that perforin mRNA expression at day 8 of gestation lacked the expected bimodal distribution usually associated with early embryo loss. In the present study, perforin mRNA expression displayed a normal distribution which appeared to be constitutively high among all the individual feto-placental units assayed. Given that previous *in vitro* assays have revealed minimal cytotoxic activity by decidual NK-cells (Baines *et al.*, 1997), it was not unexpected that potentially resorbing embryos in this study did not express significantly increased levels of perforin mRNA. Although early embryo loss has been proposed to be mediated by activated decidual NK cells, it is possible that perforin positive NK-like cells display a broad spectrum of activity and that only a few of these cells may be actively involved in embryo-toxic activities. If both resting and activated decidual NK cells expressed the same relative amounts of perforin mRNA per cell, little difference would be observed in perforin mRNA expression when comparing resorbing and potentially resorbing embryo implantation sites. Alternatively, resorption-mediating decidual NK cells may have been masked by the presence of a larger population of cells also expressing similar levels of perforin. Supporting the latter hypothesis is the recent discovery of a population of granulated lymphocytes that transiently populate the uterine mesometrial triangle in early murine pregnancy, following decidualization, and which have been shown to contain cytoplasmic granules filled with perforin (Parr *et al.*, 1990; Whitelaw and Croy, 1996). Phenotypically characterized as NK-lineage cells, these granulated metrial gland cells

(GMG) have been shown to express NK cell surface markers such as asialo-GM1, and differ from resorption-inducing decidual NK cells in that their presence is not detrimental to pregnancy. In fact, the presence of abundant numbers of GMG cells in the deciduum of pregnant mice has been shown to be essential for the normal progression of gestation (Guimond *et al.*, 1998). Previously, histological studies of perforin-expressing cells during spontaneous early embryo loss have indicated that the distribution of perforin positive GMG cells is essentially the same in resorbing and healthy decidua (Zheng *et al.*, 1993). Consistent with the data presented in this study, as well as with the contention that resorption is not caused by GMG cells and perforin-mediated cytotoxicity, this last point does not correspond with experiments that have associated early embryonic demise with increased decidual infiltration by asialo-GM1 positive cells (Gendron and Baines, 1988). This may in part be explained by the suggestion that GMG expression of asialo GM1 and many other cell surface markers may be cell stage related and transient. Indeed, GMG cell differentiation has been proposed to occur along a specific pathway, maturing from small lymphocyte-like cells to large granulated cells by mid gestation (approximately day 10-14 in mice) and altering the cell surface phenotype along the way (Parr *et al.*, 1990). In early embryo loss, increased decidual infiltration by NK cells has been proposed to occur by day 8 of gestation, a time when GMG cells may not yet express detectable levels of asialo GM1. As complete characterization of decidual NK-like cells and their biological roles have yet to be fully determined, studies aimed at determining the difference between resorption inducing NK cells and GMG cells will certainly prove to be valuable for investigating the role of NK-lineage cells during normal gestation and premature pregnancy loss.

The curious lack of perforin-mediated NK cytotoxicity during early embryo loss suggests that the decidual NK cells may have other roles to play in embryo loss. The primary role of decidual NK cells may be to mediate resorption by producing cytokines that alter the activation-state of decidual macrophages and other effector cells. Since a role for IFNγ has been well established in early embryo loss, and NK cells have been shown to produce it upon activation, this study also investigated whether NK cells were the producers of decidual IFNγ mRNA. As CBA/J mice lack NK cell specific surface markers such as NK1.1, a newly developed monoclonal anti-NK cell antibody (DX5) enabled the specific labeling of pooled decidual NK cells, *in vitro*. This experiment was performed twice and demonstrated that decidual NK cells could be the major source of decidual IFNγ as only decidual DX5 positive cells were shown to express IFNγ mRNA at day 9 of gestation. On the other hand, both DX5 positive NK cells and DX5 negative splenic cells expressed IFNγ mRNA. The DX5 negative population from the spleen would largely consist of mature T cells, which can produce IFNγ (Young *et al.*, 1995). These observations, consistent with the proposed conceptual model of early embryo loss, were also in accord with previous studies that have revealed very few conventional T lymphocytes at the fetomaternal interface (Gendron and Baines, 1988).

In this and in previous studies, simultaneous elevation of IFNγ, TNFα, and iNOS mRNA expression has been demonstrated in a portion of embryos at day 8 of gestation (Haddad *et al.*, 1997a). By demonstrating that decidual NK cells could be the major producers of the IFNγ that activates macrophage production of TNFα and NO, this study supported the premise that NK cells and macrophages act synergistically in the mechanism that causes early embryo loss in DBA/2-mated CBA/J mice. It appears that

between day 6 and day 8 of gestation, some yet to be determined initiating stimulus may be affecting the fate of embryo survival. What causes increased NK cell and macrophage infiltration, activation, and triggering of effector activity in one embryo and not in another is yet unknown and should be addressed by future work. The early presence of NK cells and macrophages detected in this study, implicated an endogenous event as the primary cause of early embryo loss in DBA/2-mated CBA/J mice. Furthermore, factors that effect the balance of TH1:TH2/3 cytokines at the feto-maternal interface have been proposed as initiating signals that predispose embryos to early resorption (Wegmann *et al.*, 1993). TH2/3 cytokines such as IL-10 and IL-4 may be beneficial to pregnancy (Chaouat *et al.*, 1995), as they prevent the stimulation of TH1 pro-inflammatory cytokines such as IFNγ, TNFα, IL-2 and IL-12. Although previous studies have shown that IL10 and immunosuppressive molecules such as TGFβ are expressed at lower levels in resorption-prone placentae, expression of these mediators at the single embryo level has not been examined. Future work should investigate the expression of anti-inflammatory cytokines at the single embyo level, with the aim detecting the earliest correlation between their decreased expression and early embryo demise. Il-12 is a switch cytokine that favors the expression of TH1 cytokines while suppressing the expression of TH2 anti-inflammatory cytokines. Recently, macrophage production of IL-12 has been associated with embryos that express elevated levels of IFNγ, TNFα, and iNOS mRNA at day 8 of gestation (Haddad *et al.*, 1997a). Future work should address the earliest expression of activating cytokines such as IL-12, IL-2 as possible correlates of early embryo loss. Furthermore, although semi-quantitative gene expression *in vitro* analyses have provided great insight into the mechanism that predisposes embryos to

early loss, future studies should also employ molecular techniques such as *in situ* hybridization, in order to specifically localize the increased TH1 gene expression patterns with the NK cells and macrophages that infiltrate the decidua-trophoblast interface during early embryo development. This technique may also enable the identification of the cells being targeted by activated macrophages and cytotoxic effector factors such as TNFα and nitric oxide. Some studies have implicated placental cells and not embryonic cells as the final targets. More recent studies have implicated a role for TNF and nitric oxide in triggering thrombosis and inflammation in the vasculature that surrounds the developing conceptus (Clark *et al.*, 1998). Future studies should attempt to determine the final damaging event that lead embryos to early death.

In conclusion, the results presented in this thesis demonstrated the correlation between implantation/early gestation and inflammatory cytokine mRNA expression. The results also support the premise that some yet to be determined endogenous factor activates the non-specific components of the maternal immune system (macrophages, NK cells and TH1 pro-inflammatory cytokines) making them critical factors in deciding between embryo death and survival. This study also showed that NK cells could be the major source of decidual IFNγ. Analysis of the molecular and cellular immunological factors that mediate early embryo loss in murine systems may eventually lead to novel concepts for the development of therapeutic treatments for women who habitually abort their babies early in pregnancy. As early embryo loss in humans tends to occur unnoticed by the maternal host, these studies may also have implications for couples with primary infertility.

References

Arck, P.C., and Clark, D.A. (1997) Immunobiology of the decidua. Curr. Topics in Microbiology and Immunology. **222**:45.

Arck, P.C., Dietl, J., Clark, D.A. (1999) From the decidual cell internet: trophoblast-recognizing T cells. Biology of Reproduction. **60**:227.

Arck, P.C., Troutt, A.B., Clark, D.A. (1997) Soluble receptors neutralizing TNF alpha and IL-1 block stress-triggered murine abortion. American Journal of Reproductive Immunology. **37**:262.

Armstrong, D.T. and Chaouat, G. (1989) Effects of lymphokines on murine placental growth in vitro. Biology of Reproduction. **39**:466.

Athanassakis, I., Bleackley, R.C., Paetkau, V., Guilbert, L., Barr, P.J., Wegmann, T.G. (1987) The immunostimulatory effect of T cells and T cell lymphokines on murine fetally-derived placental cells. Journal of Immunology. **138**:37.

Athanassakis, I., Chaouat, G., Wegmann, T.G. (1990) The effects of anti-CD4 and anti-CD8 antibody treatment on placental growth and function in allogeneic and syngeneic murine pregnancy. Cell. Immunol. **129**:13.

Athanassakis-Vassiliadis, I. and Papamettheakis, J. (1991) Modulation of class II antigens on fetal placenta leads to fetal abortion. *In* Cellular and molecular biology of the maternal-fetal relationship. Chaouat, G. and Mowbray, J.F. eds. (INSERM/John Libbey Eurotext, Paris), pp.69-81.

Baines, M.G., and Gendron, R.L. (1990) Are both endogenous and exogenous factors involved in spontaneous fetal abortion? Res. Immunol. **141**:154.

Baines, M.G., and Gendron, R.L. (1993) Natural and experimental animal models of reproductive failure. *In* Immunology of pregnancy. G. Chaouat, ed. (Boca Raton:CRC Press, Inc.), pp.173-203.

Baines, M.G., Duclos, A.J., Antecka, E., Haddad, E.K. (1997) Decidual infiltration and activation of macrophages leads to early embryo loss. American Journal of Reproductive Immunology. **37**:471.

Baines, M.G., Duclos, A.J., deFougerolles, A.R., Gendron, R.L. (1996) Immunological prevention of spontaneous early embryo resorption is mediated by non-specific immunosimulation. American Journal of Reproductive Immunology. **35**:34.

Beaman, K.D. (1990) Nidation, tolerance and immunotrophism. American Journal of Reproductive Immunology. **23**:54.

Beer, A.E., Quebbeman, J.F., Ayers, J.W., Haines, R.F. (1981) Major histocompatibility complex antigens, maternal and paternal immune responses, and chronic habitual abortions in humans. American Journal of Obstetrics and Gynecology. **141**:987.

Benirschke, K. and Robb, J.A. (1987) Infectious causes of fetal death. Clin. Obstet. Gynecol. **30**:284.

Billingham, R.E. (1964) Transplantation immunity and the maternal-fetal relation. New England Journal of Medicine. **270**:667.

Born, W., Happ, M.P., Dallas, A., Reardon, C., Kubo, R., Shinnick, T., Brennan, P., O'Brien, R. (1990) Recognition of heatshock proteins and gamma delta cell function. Immunology Today. **11**:40.

Caccavo, D., Vaccaro, F., Ferri, G.M., Amoroso, A., Bonomo, L. (1994) Anti-idiotypes against antiphospholipid antibodies are present in normal polyspecific immunoglobulins for therapy use. Journal of Autoimmunity. **7**:537.

Carosella, E.D., Rouas-Freiss, N., Paul, P., Dausset, J. (1999) HLA-G: a tolerance molecule from the major histocompatibility complex. Immunology Today. **20**:60.

Carp, H.J.A., Toder, V., Gazit, E., Orgad, S., Mashiach, S., Nebel, L., Serr, D.M. (1990) Immunization by paternal leukocytes for prevention of primary habitual abortion-results of a matched controlled trial. Gynecol. Obstet. Invest. **29**:16.

Chaouat, G. (1993) The root of the problem: "the fetal allograft". *In* Immunology of pregnancy. G. Chaouat, ed. (Boca Raton:CRC Press, Inc.), pp.1-17.

Chaouat, G., Clark, D.A., Wegmann, T.G. (1988) Genetic aspects of the CBA/J x DBA/2 and B10 x B10.A models of murine spontaneous abortions and prevention by leukocyte immunization. *In*: Early Pregnancy Loss: Mechanisms and Treatments. Allen, W.R., Clark, D.A., Gill, T. J., Mowbray, J.F., Robertson, W.R. eds. (RCOG Press, London), pp.89-109.

Chaouat, G. and Lankar, D. (1988) Vaccination against spontaneous abortion in mice by preimmunization with an anti-idiotypic antibody. American Journal of Reprod. Immunol. Microbiol. **16**:146.

Chaouat, G., Meliani, A.A., Martal, J., Raghupathy, R., Elliot, J., Mosmann, T., Wegmann, T.G. (1995) IL-10 prevents naturally occurring fetal loss in the CBA x

DBA/2 mating combination, and local defect in IL-10 production in this abortion-prone combination is corrected by *in vivo* injection of IFN-τ. Journal of Immunology. **154**:4261.

Chaouat, G. and Menu, E. (1997) Maternal T cell reactivity in pregnancy? Current Topics in Microbiology and Immunology. **222**:103.

Chaouat, G., Menu, E., Clark, D.A., Dy, M., Minkowski, M., Wegmann, T.G. (1990) Control of fetal survival in CBA x DBA/2 mice by lymphokine therapy. Journal of Reprod. Fertil. **89**:447.

Chen, H.L., Kamath, R., Pace, J.L., Rusell, S.W., Hunt, J.S. (1994) Gestation-related expression of the interferon-gamma receptor gene in mouse uterine and embryonic hematopoietic cells. Journal of Leukocyte Biology. **55**:617.

Chen, H-L., Yang, Y., Hu, X-L., Yelavarthi, K.K., Fishback, J.L., Hunt, J.S. (1991) Tumor necrosis factor alpha mRNA and protein are present in human placental and uterine cells at early and late stages of gestation. American Journal of Pathology. **139**:327.

Chomczynski, P. and Sacchi, N. (1987) Single step method of RNA isolation by acid guanidine thiocyanate phenol chloroform extraction. Journal Anal. Biochem. **162**:156.

Clark, D.A. (1993) Uterine cells of immunologic relevance in animal systems. *In* Immunology of pregnancy. G. Chaouat, ed. (Boca Raton:CRC Press, Inc.), pp.79-92.

Clark, D.A., Arck, P.C., Jalali, R., Merali, F.S., Manuel, J., Chaouat, G., Underwood, J.L., Mowbray, J.F. (1996) Psycho-neuro-cytokine/endocrine

pathways in immunoregulation during pregnancy. American Journal of Reproductive Immunology. **35**:330.

Clark, D.A., Quarrington, C., Banwatt, D., Manuel, J., Fulop, G. (1994) Spontaneous abortion in immunodeficient SCID mice. American Journal of Reproductive Immunology. **32**:15.

Clark, D.A. and Chaouat, G. (1989) What do we know about spontaneous abortion mechanisms? American Journal of Reproductive Immunology. **19**:28.

Clark, D.A., Chaouat, G., Arck, P.C., Mittruecker, H.W., Levy, G.A. (1998) Cutting edge: cytokine-dependent abortion in CBA x DBA/2 mice is mediated by the procoagulant fgl2 prothombinase. Journal of Immunology. **160**:545.

Clark, D.A. and Coulam, C.B. (1996) Is there an immunological cause of repeated preganacy wastage? Adv. Obstet. Gynecol. **3**:321.

Clark, D.A., McDermott, M.R., Szewczuk, M.R. (1980) Impairment of host-vs-graft reaction of pregnant mice. II. Selective suppression of cytotoxic T-cell generation correlates with successful allogeneic pregnancy. Cell. Immunol. **52**:106.

Clark, D.A., Merali, F.S., Hoskin, D.W., Steel-Norwood, D., Arck, P.C., Croitoru, K., Murgita, R.A., Hirte, H. (1997) Decidua-associated suppressor cells in abortion-prone DBA/2-mated CBA/J mice that release bioactive transforming growth Factor β2-related immunosuppessive molecules express a bone marrow-derived natural suppressor cell marker and γδ T-cell receptor. Biology of Reproduction. **56**:1351.

Coulam, C.B., Stephenson, M., Stern, J.J., Clark, D.A. (1996) Immunotherapy for recurrent pregnancy loss: analysis of results from clinical trials. American Journal of Reproductive Immunology. **35**:352.

Crawford, R.M., Leiby, D.A., Green, S.J., Nacy, C.A., Fortier, A.H., Meltzer, M.S. (1994) Macrophage activation: a riddle of immunological resistance. Immunol. Ser. **60**:29.

Croy, B.A., Ashkar, A.A., Foster, R.A., Disanto, J.P., Magram, J., Carson, D., Gendler, S.J., Grusby, M.J., Wagner, N., Müller, W., Guimond, M.J. (1997) Histological studies of gene-ablated mice support important functional roles for natural killer cells in the uterus during pregnancy. Journal of Reproductive Immunology. **35**:111.

Croy, B.A. and Kiso, Y. (1993) Granulated metrial gland cells: a natural killer cell subset of the pregnant murine uterus. Microsc. Res. Tech. **25**:189.

Croy, B.A., Reed, N., Malashenko, B.A., Kim, K., Kwon, B.S. (1991) Demonstration of YAC target cell lysis by murine granulated metrial gland cells. Cellular Immunology. **133**:116.

DaSilva, J.A. and Spector, T.D. (1992) The role of pregnancy in the course and etiology of rheumatoid arthritis. Clin. Rheumatol. **11**:189.

Daya, S., Gunby, J., Clark, D.A. (1998) Intravenous immunoglobulin therapy for recurrent spontaneous abortion: a meta-analysis. American Journal of Reproductive Immunology. **39**:69.

deFougerolles, A.R. and Baines, M.G. (1997) Modulation of the natural killer cell activity in pregnant mice alters the spontaneous abortion rate. Journal of Reproductive Immunology. **11**:147.

Ding, A.H., Nathan, C.F., Stuehr, D.J. (1988) Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. Comparison of activating cytokines and evidence for independent production. Journal of Immunology. **141**:2407.

Drake, B.L. and Head, J.R. (1989) Murine trophoblast can be killed by lymphokine-activated killer cells. Journal of Immunology. **143**:9.

Duclos, A.J., Haddad, E.K., Baines, M.G. (1995) Presence of activated macrophages in a murine model of early embryo loss. American Journal of Reproductive Immunology. **33**:354.

Duclos, A.J., Haddad, E.K., Chalifour, L.E., Baines, M.G. (1996) Embryo infiltration by maternal macrophages is associated with selective expression of proto-oncogenes in a murine model of spontaneous abortion. Biology of Reproduction. **54**:1088.

Duclos, A.J., Pomerantz, D.K., Baines, M.G. (1994) Relationship between decidual leukocyte infiltration and spontaneous abortion in a murine model of early fetal resorption. Cell. Immunol. **159**:184.

Edmonds, D.K., Lindsay, K.S., Miller, J.F., Williamson, E. and Wood, P.J. (1982) Early embryonic mortality in women. Fert. Steril. **34**:447.

Eischen, C.M., Schilling, J.D., Lynch, D.H., Krammer, P.H., Leibson, P.J. (1996) Fc receptor-induced expression of fas ligand on activated NK cells facilitates cell-mediated cytotoxicity and subsequent autocrine NK cell apoptosis. Journal of Immunology. **156**:2693.

Enders, A.C. (1991) Current topics: structural response of the primate endometrium to implantation. Placenta. **12**:309.

Faulk, W.P. and Hunt, J.S. (1990) Human trophoblast antigens. Immunol. Allergy Clin. N. Am. Reprod. Immunol. **10**(1): 27.

Gafter, U., Sredni, B., Segal, J., Kalechman, Y. (1997) Suppressed cell-mediated immunity and monocyte and natural killer cell activity following allogeneic immunization of women with spontaneous recurrent abortion. Journal of Clin. Immunol. **17**:408.

Gendron, R.L. and Baines, M.G. (1988) Infiltrating decidual natural killer cells are associated with spontaneous abortion in mice. Cell. Immunol. **113**:261.

Gendron, R.L., Nestel, F.P., Lapp, W.S., Baines, M.G. (1990) Lipopolysaccharide induced fetal resorption in mice is associated with intrauterine production of tumour necrosis factor-alpha. Journal of Reprod. Fertil. **90**:395.

Gendron, R.L., Nestel, F.P., Lapp, W.S., Baines, M.G. (1991) Expression of tumor necrosis factor alpha in the developing nervous system. Int. J. Neurosci. **60**:129.

Gill, T.J.III., Ho, H-N., Kunz, H.W. (1993) Immunogenetic of reproduction. *In* Immunology of pregnancy. G. Chaouat, ed. (Boca Raton:CRC Press, Inc.), pp.19-31.

Gorivodsky, M., Zemlyak, I., Orenstein, H., Savion, S., Fein, A., Torchinsky, A., Toder, V. (1998) TNFα messenger RNA and protein expression in the uteroplacental unit of mice with pregnancy loss. Journal of Immunology. **160**:4280.

Griffith, S. and Fergusin, T.A. (1997) The role of FasL-induced apoptosis in immune privilege. Immunology Today. **18**:240.

Guimond, M.J., Luross, J.A., Wang, B., Terhorst, C., Danial, S., Croy, B.A. (1997) Absence of natural killer cells during murine pregnancy is associated with reproductive compromise in Tgε26 mice. Biol. Reprod. **56**:169.

Guimond, M.J., Wang, B., Croy, B.A. (1998) Engraftment of bone marrow from severe combined immunodeficient (SCID) mice reverses the reproductive deficitsin natural killer cell-deficient tgε26 mice. Journal of Experimental Medicine. **187**:217.

Haddad, E.K., Duclos, A.J., Antecka, E., Lapp, W.S., Baines, M.G. (1997a) Role of interferon-γ in the priming of decidual macrophages for nitric oxide production and early embryo loss. Cell. Immunol. **181**:68.

Haddad, E.K., Duclos, A.J., Baines, M.G. (1995) Early embryo loss is associated with local production of nitric oxide by decidual mononuclear cells. Journal of Experimental Medicine. **182**:1143.

Haddad, E.K., Duclos, A.J., Lapp, W.S., Baines, M.G. (1997b) Early embryo loss is associated with prior expression of macrophage activation markers in the decidua. Journal of Immunology. **158**:4886.

Hafez, E.S.E. (1984) Spontaneous abortion. Adv. Reprod. Health care. **1**:1.

Hammer, A., Blaschitz, A., Daxbock, C., Walcher, W., Dohr, G. (1999) Fas and Fas- ligand are expressed in the uteroplacental unit of first-trimester pregnancy. American Journal of Reproductive Immunology. **41**:41.

Heyborne, K., Fu, Y-X., Nelson, A., Farr, A., O'Brien, R., Born, W. (1994) Recognition of trophoblasts by γδ T cells. Journal of Immunology. **153**:2918.

Hill, J.A. (1991) Implications of cytokines in male and female sterility. *In* Cellular and molecular biology of the maternal-fetal relationship. Chaouat, G. and Mowbray, J.F. eds. (INSERM/John Libbey Eurotext, Paris), pp.269-275.

Hill, J.A. (1992) Cytokines considered critical in pregnancy. American Journal of Reproductive Immunology. **28**:123.

Hill, J.A., Anderson, D.J., Polgar, K. (1995) T helper 1-type cellular immunity to trophoblast in women with recurrent spontaneous abortions. JAMA. **273**:1933.

Hill, J.A. and Ravinikar, V.A. (1990) Recurrent abortion. *In* Kistner's Gynecology: Principles and Practice. Ryan, K.J., Berkowitz, R., Barbieri, R.L. eds. (Chicago USA, Yearbook Medical Publishers), pp.406.

Holland, D., Bretscher, P., Russel, A.S. (1984) Immunologic and inflammatory responses during pregnancy. Clin. Lab. Immunol. **14**:177.

Hunt, J.S. (1994) Immunologically relevant cells in the uterus. Biology of Reproduction. **50**:461.

Hunt, J.S., Chen, H.L., Miller, L. (1996) Tumor necrosis factors: pivotal components of pregnancy? Biology of Reproduction. **54**:554.

Hunt, J.S., Fishback, J.L., Andrews, G.K., Wood, G.W. (1988) Expression of class I HLA genes by trophoblast cells: analysis by in situ hybridization. Journal of Immunology. **140**:1293.

Hunt, J.S., Manning, L.S., Mitchell, D., Selanders, J.R., Wood, G.W. (1985) Localization and characterization of macrophages in murine uterus. Journal of Leukocyte Biology. **38**:255.

Hunt, J.S., Miller, L., Roby, K.F., Huang, J., Platt, J.S., DeBrot, B.L. (1997a) Female steroid hormones regulate production of pro-inflammatory molecules in uterine leukocytes. Journal of Reproductive Immunology. **35**:87.

Hunt, J.S., Miller, L., Vassmer, D., Croy, A.B. (1997b) Expression of the inducible nitric oxcide synthase gene in mouse uterine leukocytes and potential relationships with uterine function during pregnancy. Biology of Reproduction. **57**:827.

Hunt, J.S. and Robertson, S.A. (1996) Uterine macrophages and environmental programming for pregnancy success. Journal of Reprod. Immunol. **32**:1.

Hunt, J.S., Vassmer, D., Ferguson, T.A., Miller, L. (1997c) Fas ligand is positioned in mouse uterus and placenta to prevent trafficking of activated leukocytes between the mother and the conceptus. Journal of Immunology. **158**:4122.

Jalali, G.R., Arck, P., Surridge, S., Markert, U., Chaouat, G., Clark, D.A., Underwood, J.L., Mowbray, J.F. (1996) Immunosuppressive properties of monoclonal antibodies to the R80K protein of trophoblast. American Journal of Reproductive Immunology. **36**:129.

Jalali, G.R., Underwood, J.L., Mowbray, J.F. (1989) IgG on normal human placenta is bound to antigen and Fc receptors. Transplant Proc. **2**:572.

Jameson, E.M. (1935) Gynecological and Obstetrical Tuberculosis, Lea and Febiger.

Jiang, S.P. and Vacchio, M.S. (1998) Cutting edge: Multiple mechanisms of peripheral T cell tolerance to the fetal "allograft". The Journal of Immunology. **160**:3086.

Kiger, N., Chaouat, G., Kolb, J.P., Wegmann, T.G., Guenet, J.L. (1985) Immunogenetic studies of spontaneous abortion in mice: preimmunization of females with allogeneic cells. Journal of Immunology. **134**:2966.

King, A., Loke, Y.W., Chaouat, G. (1997) NK cells and reproduction. Immunology Today. **18**:64.

Knowles, R.G. and Moncada, S. (1994) Nitric oxide synthases in mammals. Biochem. J. **298**:249.

Krishnan, L., Guilbert, L.J., Wegmann, T.J., Belosevic, M., Mosmann, T.R. (1996) T helper 1 response against *Leishmania major* in pregnant C57BL/6 mice increases implantation failure and fetal resorptions. Journal of Immunology. **156**:653.

King, A. and Loke, Y.W. (1991) On the nature and function of human uterine granulated lymphocytes. Immunology Today. **12**:432.

Kinsky, R., Delage, G., Rosin, N., Thang, M.N., Hoffmann, M., Chaouat, G. (1990) A murine model of NK cell mediated resorption. American Journal of Reproductive Immunology. **23**:73.

Lachapelle, M.H., Miron, P., Hemmings, R., Roy, D.C. (1996) Endometrial T, B, and NK cells in patients with recurrent spontaneous abortion. Journal of Immunology. **156**:4027.

Lanier, L.L. and Phillips, J.H. (1996) Inhibitory MHC class I receptors on NK cells and T cells. Immunology Today. **17**:86.

Lea, R.G., Flanders, K.C., Harley, C.B., Manuel, J., Banwatt, D., Clark, D.A. (1992) Release of a TGF-β2-related suppressor factor from post-implantation murine

decidual tissue can be correlated with the detection of a subpopulation of cells containing RNA for TGF-β2. Journal of Immunology. **148**:778.

Lea, R.G., Underwood, J., Flanders, K.C., Hirte, H., Banwatt, D., Finotto, S., Ohno, I., Daya, S., Harley, C., Michel, M., Mowbray, J.F., Cark, D.A. (1995) A subset of patients with recurrent spontaneous abortion is deficient in transforming growth factor β2-producing "suppressor cells" in uterine tissue near the placental attachment site. American Journal of Reproductive Immunology. **34**:52.

Liles, W.C., Kiener, P.A., Ledbetter, J.A., Arufo, A., Klebanoff, S.J. (1996) Differential expression of Fas (CD95) and FasL on normal human phagocytes: implications for the regulation of apoptosis in neutrophils. Journal of Experimental Medicine. **184**:429.

Lippman, A. and Farookhi, R. (1986) The Montreal Pregnancy Study: an investigation of very early pregnancies. Can. J. Pub. Health. **77suppl1**:157.

Liu, C.-C., Parr, E.L., Young, J.D.-E. (1994) Granulated lymphoid cells of the pregnant uterus: morphological and functional features. Int. Rev. Cytol. **153**:105.

Liu, C.-C., Walsh, C.M., Young, J.D.E. (1995) Perforin: structure and function. Immunology Today. **16**:194.

Luft, B.J. and Remington, J.S. (1982) Effect of pregnancy on resistance to *Listeria monocytogenes* and *Toxoplasma gondii* infections in mice. Infection and Immunity. **38**:1164.

Luft, B.J. and Remington, J.S. (1984) Effects of pregnancy on augmentation of natural killer cell activity by *Corynebacterium parvum* and *Toxoplasma gondi.* Journal of Immunology. **132**:2375.

McLaughlin, J. (1990) Human listeriosis in Britain, 1967-85, a summary of 722 cases. 2. Listeriosis in non-pregnant individuals, a changing pattern of infection and seasonal incidence. Epidemiology and Infection. **104**:191.

Menu, E., Chaouat, G., Kinsky, R., Delage, G., Kapovic, M., Thang, M.N., Jaulin, C., Kourilsky, P., Wegmann, T.G. (1995) Alloimmunization against well defined polymorphic major histocompatibility or class I MHC transfected L cells antigens can prevent poly IC induced fetal death in mice. American Journal of Reproductive Immunology. **33**:200.

Miller, L. and Hunt, J.S. (1998) Regulation of TNFα production in activated mouse macrophages by progesterone. Journal of Immunology. **160**:5098.

Moncada, S., Palmer, R.M., Higgs, E.A. (1991) Nitric oxide physiology, pathophysiology, and pharmacology. Pharmacol. Rev. **43**:109.

Mowbray, J.F., Gibbings, C., Liddell, H., Reginald, P.W., Underwood, J.L., Beard, R.W. (1985) Controlled trial of recurrent spontaneous abortion by immunization with paternal cells. Lancet. **1**:941.

Mowbray, J., Jalali, R., Chaouat, G., Clark, D.A., Underwood, J., Allen, W.R., Mathias, S. (1997) Maternal Response to Paternal Trophoblast Antigens. American Journal of Reproductive Immunology. **37**:421.

Mowbray, J.F., Underwood, J.L., Jalali, G.R. (1993) Immunological processes of abortion. *In* Immunology of Pregnancy. G. Chaouat, ed. (Boca Raton: CRC Press, Inc.), pp:231-244.

Munoz-Fernandez, M.A., Fernandez, M.A., Fresno, M. (1992) Synergism between tumour necrosis factor-alpha and interferon-gamma on macrophage

activation for the killing of intracellular *Trypanosoma cruzi* through nitric oxide-dependent mechanism. European Journal of Immunolgy. **22**:301.

Nagata, S. and Golstein, P. (1995) The Fas death factor. Science. **267**:1449.

Ortaldo, J.R., Mason, L.H., Gregorio, T.A., Stoll, J., Winkler-Pickett, R.T. (1997) The Ly-49 family: regulation of cytokine production in murine NK cells. Journal of Leukocyte Biology. **62**:381.

Parr, E.L., Parr, M.B., Young, J.D. (1987) Localization of a pore-forming protein (perforin) in granulated metrial gland cells. Biology of Reproduction. **37**:1327.

Parr, E.L., Young, L.H.Y., Parr, M.B., Young, J.D-E. (1990) Granulated metrial gland cells of pregnant mouse uterus are natural killer-like cells that contain perforin and serine esterases. The Journal of Immunology. **145**:2365.

Peters, P.M., Ortaldo, J.R., Shalaby, M.R., Svedersky, L.P., Nedwin, G.E., Bringman, T.S., Hass, P.E., Aggarwal, B.B., Berberman, R.B., Goeddel, D.V., Palladino, M.A. (1986) NK-sensitive targets stimulate production of TNF-α but not TNF-β by highly purified human peripheral blood large granular lymphocytes. Journal of Immunology. **137**:2592.

Platt, J.S. and Hunt, J.S. (1998) Interferon-γ gene expression in cycling and pregnant mouse uterus: temporal aspects and cellular localization. Journal of Leukocyte Biology. **64**:393.

Pollard, J.W., Hunt, J.S., Wiktor-Jedrzejczak, W., Stanley, E.R. (1991) A pregnancy defect in the osteopetrotic *(op/op)* mouse demonstrates the requirement for CSF-1 in female fertility. Dev. Biol. **148**:273.

Raghupathy, R. (1997) Maternal anti-placental cell-mediated reactivity and spontaneous abortion. American Journal of Reproductive Immunology. **37**:478.

Raghupathy, R. and Tangri, S. (1996) Immunodystrophism, T cells, cytokines, and pregnancy failure. American Journal of Reproductive Immunology. **35**:291-296.

Rajagopalan, S. and Long, E.O. (1999) A human histocompatibility leukocyte antigen (HLA)-G-specific receptor expressed on all natural killer cells. Journal of Experimental Medicine. **189**:1093.

Redline, R.W. and Lu, C.Y. (1987) Role of local immunosuppression in murine fetoplacental listeriosis. Journal of Clin. Invest. **79**:1234.

Redline, R.W. and Lu, C.Y. (1988) Specific defects in the anti-listerial immune response in discrete regions of the murine uterus and placenta account for susceptibility to infection. Journal of Immunology. **140**:3947.

Robertson, S.A., Roberts, C.T., Farr, K.L., Dunn, A.R., Seamark, R.F. (1999) Fertility impairment in granulocyte-macrophage colony-stimulating factor-deficient mice. Biology of Reproduction. **60**:251.

Robaye, B., Mosselmans, R., Fiers, W., Dumont, J.E., Galand, P. (1991) Tumor necrosis factor induces apoptosis (programmed cell death) in normal endothelial cells in vitro. Am. J. Pathol. **138**:447.

Roby, K.F. and Hunt. J.S. (1994) Mouse endometrial tumor necrosis factor-α mRNA and protein localization and regulation by estradiol and progesterone. Endocrinology. **135**:2780.

Roby, K.F., Laham, N., Kroning, H., Terranova, P.F., Hunt, J.S. (1995) Expression and localization of messenger RNA for tumor necrosis factor receptor (TNF-R) I and TNF-RII in pregnant mouse uterus and placenta. Endocrine. **3**:557.

Roth, I., Corry, D.B., Locksley, R.M., Abrams, J.S., Litton, M.J., Fisher. S.J. (1996) Human placental cytotrophoblasts produce the immunosuppressive cytokine interleukin 10. Journal of Experimental Medicine. **184**:539.

Rouas-Freiss, N., Marchal, R.E., Kirszenbaum, M., Dausset, J., Carosella, E.D. (1997) The alpha1 domain of HLA-G1 and HLA-G2 inhibits cytotoxicity induced by natural killer cells: is HLA-G the public ligand for natural killer cell inhibitory receptors? Proc. Natl. Acad. Sci. USA. **94**:5249.

Rouvier, E., Luciani, M.F., Golstein, P. (1993) Fas involvement in Ca(2+)-independent T cell-mediated cytotoxicity. Journal of Experimental Medicine. **177**:195.

Sacks, G., Sargent., I., Redman, C. (1999) An innate view of pregnancy. Immunology Today. 20:114.

Saito, S., Nishikawa, K., Morii, T., Enomoto, M., Narita, N., Motoyoshi, K., Ichijo, M. (1993) Cytokine production by CD16-CD56 bright natural killer cells in the human early pregnancy decidua. Int. Immunol. **5**:559.

Saito, S., Nishikawa, K., Morii, T., Narita, N., Enomoto, M., Ito, A., Ichijo, M. (1994) A study of CD45RO, CD45RA and CD29 antigen expression on human decidual T cells in an early stage of pregnancy. Immunol. Letters. **40**:193.

Saji, F. (1993) Late trophoblast immunoregulatory factors. *In* Immunology of pregnancy. G. Chaouat, ed. (Boca Raton:CRC Press, Inc.), pp.125-141.

Sidhu, R.S. and Bollon, A.P. (1993) Tumor necrosis factor activities and cancer therapy- a perspective. Pharmacol. Ther. **57**:79.

Southern, E.M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. Journal of Mol. Biol. **98**:503.

Stern, J.J., Darfmann, A.D., Gutierrez-Nagar, A.J., Cerrillo, M., Coulam, C. (1996) Frequency of abnormal karyotypes among abortuses from women with and without a history of recurrent spontaneous abortion. Fertil. Steril. **65**:250.

Stray, B. and Stray-Pederson, S. (1984) Etiological factors and subsequent reproductive performance in 195 couples with a prior y of habitual abortion. American J. Obstet. Gynecol. **148**:140.

Suzuki, T., Hiromatsu, K., Ando, Y., Okamoto, T., Tomoda, Y., Yosjikai, Y. (1995) Regulatory role of γδ T cells in uterine intraepithelial lymphocytes in maternal antifetal immune response. Journal on Immunology. **154**:4476.

Szekeres-Bartho, J., Kinsky, R., Chaouat, G. (1990) The effect of a progesterone-induced immunologic blocking factor on NK-mediated resorption. American Journal of Immunology. **24**:105.

Szekeres-Bartho, J., Kinsky, R., Kapovic, M., Chaouat, G. (1991) Complete Freund adjuvant treatment of pregnant females influences resorption rates in CBA x DBA/2 matings via progesterone-mediated immunomodulation. American Journal of Reproductive Immunology. **26**:82.

Szekeres-Bartho, J., Reznikoff-Etievant, M.F., Varga, P., Pichon, M.F., Varga, Z., Chaouat, G. (1989) Lymphocytic progesterone receptors in normal and pathological human pregnancy. Journal of Reproductive Immunology. **16**:239.

Szekeres-Bartho, J. and Wegmann, T.G. (1996) A progesterone-dependent immunomodulatory protein alters the TH1/TH2 balance. Journal of Reproductive Immunology. **31**:81.

Tangri, S. and Raghupathy, R. (1993) Expression of cytokines in placentas of mice undergoing immunologically mediated spontaneous fetal resorptions. Biology of Reproduction. **49**:850.

Tangri, S., Wegmann, H.L., Raghupathy, R. (1994) Maternal anti-placental reactivity in natural, immunologically-mediated fetal resorptions. Journal of Immunology. **152**:4903.

Trincheri, G. (1989) Biology of natural killer cells. Adv. Immunol. **47**:187.

Torry, D.S., McIntyre, J.A., Faulk, W.P. (1997) Immunobiology of the trophoblast: mechanisms by which placental tissues evade maternal recognition and rejection: curr. Topics in Microbiology and Immunology. **222**:127.

Uckan, D., Steele, A., Cherry, Wang, B.Y., Chamizo, W., Koutsonikolis, A., Gilbert-Barness, E., Good, R.A. (1997) Trophoblasts express Fas ligand: a proposed mechanism for immune privilege in placenta and maternal invasion. Molecular Human reproduction. **3**:655.

Vassalli, P. (1992) The pathophysiology of tumor necrosis factors. Annual Review of Immunology. **10**:411.

Vassiliadou, N. and Bulmer, J.N. (1996) Immunohistochemical evidence for increased numbers of 'classic' CD56+ natural killer cells in the endometrium of women suffering spontaneous early pregnancy loss. Hum. Reprod. **11**:1659.

Vince, G., Shorter, S., Starkey, P., Humphreys, J., Clover, L., Wilkins, T., Sargent, I., Redman, C. (1992) Localization of tumor necrosis factor production in cells at the materno/fetal interface in human pregnancy. Clin. Exp. Immunol. **88**:174.

Watkinson, M. and Rushton, D.I. (1983) Plasmodial pigmentation of placenta and outcome of pregnancy in West African mothers. British Med. J. Clin. Res. **287**:251.

Wegmann, T.G. (1986) Placental immunotrophism: maternal T cells enhance placental growth and function. American Journal of Reproductive Immunology. **15**:67.

Wegmann, T.G. (1987) Immunoregulation and fetal survival. Gill, T.J.III. and Wegmann, T.G. eds. (Oxford University Press, New York).

Wegmann, T.G., Lin, H., Guilbert, L., Mosmann, T.R. (1993) Bidirectional cytokine intractions in the maternal–fetal relationship: is successful pregnancy a TH2 phenomenon? Immunology Today. **14**:353.

Whitelaw, P.F. and Croy, B.A. (1996) Granulated lymphocytes of pregnancy. Placenta. **17**:533.

Wilcox, A.J., Weinberg, C.R., O'Connor, J.F., Baird, D.D., Schlatterer, J.P., Canfield, R.E., Armstrong, E.G., Nisula, B.C. (1988) Incidence of early loss of pregnancy. New England Journal of Medicine. **319**:189.

Ye, W., Zheng, L.-M., Young, J.D.-E. (1996) The involvement of interleukin (IL) –15 in regulating the differentiation of granulated metrial gland cells in mouse pregnant uterus. Journal of Experimental Medicine. **184**:2405.

Yelavarthi, K.K., Chen, H.L., Yang, Y., Fishback, J.L., Courley, B.Jr., Hunt, J.S. (1991) Tumor necrosis factor-α mRNA and protein in rat uterine and placental cells. Journal of Immunology. **146**:3840.

Yokoyama, W. (1997) The mother-child union: The case of missing-self and protection of the fetus. Proc. Natl. Acad. Sci. **94**:5998.

Young, H.A. and Hardy, K.J. (1995) Role of interferon-gamma in immune cell regulation. Journal of Leukocyte Biology. **58**:373.

Zheng, L.M., Joag, S.V., Parr, M.B., Parr, E.L., Young, J.D.-E. (1991) Perforin-expressing granulated metrial gland cells in murine deciduoma. Journal of Experimental Medicine. **174**:1221.

Zheng, L.M., Ojcius, D.M., Young, J.D. (1993) Perforin expressing cells during spontaneous abortion. Biology of Reproduction. **48**:1014.

Zuckermann, F.A. and Head, J.R. (1988) Murine trophoblast resists cell-mediated lysis. II. Resistance to natural cell-mediated cytotoxicity. Cell. Immunol. **116**:274.