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The role of placental interleukin 10 in maternal immune suppression and the control of cytotrophoblast invasion

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The Role of Placental Interleukin 10 in Maternal Immune Suppression and the Control of Cytotrophoblast Invasion

by

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THE ROLE OF HUMAN PLACENTAL INTERLEUKIN 10 IN MATERNAL IMMUNE SUPPRESSION AND THE CONTROL OF CYTOTROPHOBLAST INVASION

by

Iris Roth

ABSTRACT

Successful human pregnancy requires the correct formation of the placenta. During placentation, extraembryonic cytotrophoblast stem cells differentiate along two distinct pathways to form an interface between fetal and maternal circulations. Mononuclear stem cells fuse to form syncytiotrophoblasts, whose primary function is to mediate nutrient, waste, and gas exchange for the developing fetus. Alternately, cytotrophoblasts differentiate to acquire an invasive phenotype, then migrate through the endometrium and disrupt maternal arteries. This process serves both to anchor the placenta to the uterine wall and to supply the fetus with nutrient-rich arterial blood. The consequence of cytotrophoblast differentiation is the mixing of genetically foreign fetal cells with large numbers of maternal cells, including leukocytes.

In light of this, the fact that the mother accepts the placenta as an allograft remains a great biological mystery. No single hypothesis appears to explain fetal evasion of the maternal immune system. Thus, it is likely that multiple protective mechanisms exist to ensure that viviparity succeeds. One of these is the localized secretion of factors that can modulate a potentially deletrious immune response. Various molecules produced by different placental cells have been shown to suppress immune function. Among them are cytokines,

pleiotropic factors that play a role in the regulation of the growth and differentiation of a variety of cell types, including those of the immune system.

Our work has focused on the characterization of cytokine secretion by cytotrophoblast cells. Using an *in vitro* system that models uterine invasion by cytotrophbolasts, we found that these cells produce interleukin (IL) -10. We provide evidence that cytotrophoblast IL-10 has two important functions *in vitro*: 1) the paracrine suppression of allogeneic T lymphoycte reactions, and 2) the autocrine inhibition of cytotrophoblast invasion. Our results suggest that cytotrophoblast secretion of IL-10 at the maternal-fetal interface *in vivo* may dampen a maternal immune response and regulate placental attachment.

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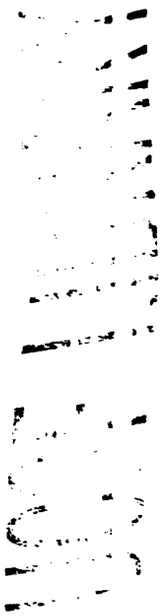
LIST OF ABBREVIATIONS

AFP	alpha-fetoprotein
APC	antigen-presenting cells
CD	cluster of differentiation
CM	conditioned medium
CMV	cytomegalovirus
CTL	cytotoxic T lymphocyte
DTH	delayed type hypersensitivity
E-cadherin	epithelial-cadherin
ECM	extracellular matrix
FcR	Fc receptor
Fn	fibronectin
hCG	human chorionic gonadotropin
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
hPL	human placental lactogen
HPRT	hypoxanthine-guanine phosphoribosyltransferase
HSV	herpes simplex virus
ICAM	intercellular adhesion molecule
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IL-10R	interleukin-10 receptor
IUGR	intrauterine growth retardation
LAK	lymphokine-activated killer cell
LFA	lymphocyte function-associated antigen
Ln	laminin
LPS	lipopolysaccharide
mAb	monoclonal antibody
MHC	major histocompatibility complex
MLR	mixed lymphocyte reaction
MMP	matrix metalloproteinase
NK	natural killer cell
PA	plasminogen activators
PAPP-A	pregnancy-associated plasma protein-A
PBL	peripheral blood leukocytes
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PECAM-1	platelet-endothelial cell adhesion molecule-1
PGE_2	prostaglandin
PSGs	pregnancy specific glycoproteins
PSTT	placental site trophoblastic tumor
rIL-10	recombinant interleukin-10;
t-PA	tissue-type plasminogen activator
TCR	T cell receptor
Th	T helper cell
TIMP	tissue inhibitor of metalloproteinases
TLX	trophoblast-leukocyte common antigen
TM	trimester
TNF	tumor necrosis factor
u-PA	urokinase-type plasminogen activator

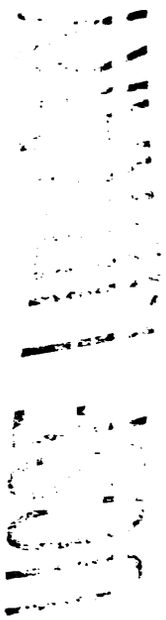
CHAPTER ONE

Development of the Human Placenta



General Introduction

Successful human pregnancy requires the correct formation of the placenta. This organ forms the interface between maternal and fetal circulations and plays the critical role of providing nutrient, waste and gas exchange for the developing embryo. Placentation is the result of the differentiation of specialized epithelial cells known as trophoblasts. The initial segregation of the trophoblast lineage, occurring during preimplantation development, can be studied using human zygotes fertilized in vitro (Lindenberg et al., 1989; Lindenberg et al., 1985). Placental tissue is also readily available from terminated pregnancies, and is useful for studying later aspects of trophoblast differentiation. Unfortunately, this material can only be collected as early as six weeks gestation, when placental organogenesis is complete. Because the window of time between implantation and pregnancy termination is inaccessible to experimental manipulation, little is known about trophoblast differentiation during formation of a mature placental structure. However, observations of several rare histological specimens of embryos from seven to seventeen days of development have provided some insight into this complex process (Boyd, 1970; Hamilton and Boyd, 1960; Hertig, 1941; Hertig, 1949; Hertig, 1945; Hertig et al., 1956), Genbacev, O., personal communication).



Placental Histology: the Previllous Stage (fertilization through two weeks)

Placentation begins when the human blastocyst implants into the uterine wall. In vitro fertilization provides material for studying the histology of the periimplantation blastocyst. At this stage of development, mural cytotrophoblasts, the cells that will form the placenta, surround the inner cell mass, which is destined to become the embryo proper (Figure 1-1A). The embryoblast is apposed to the inner surface of the trophoblast cover at the embryonic pole. Implantation is initiated when cytotrophoblasts adhere to the endometrial

epithelium sometime between days 5 and 6 after fertilization, in a process called apposition (Boyd, 1970). Blastocyst implantation and, subsequently, placental attachment, occur most often in the upper part of the posterior uterine wall. In vitro studies with monolayer cultures of uterine epithelial cells demonstrate that adhesion occurs at the embryonic pole of the blastocyst, which is also called the implantation pole (Lindenberg et al., 1986; Lindenberg et al., 1989; Lindenberg et al., 1985).

Subsequent to endometrial attachment, the conceptus begins to embed itself into the uterine stroma. The mechanism of blastocyst invasion of the epithelial cell layer in vivo is undefined, as human material at this time of development is largely lacking or poorly preserved. Transmission electron microscopy of epithelial invasion in vitro suggests that it is of the intrusive type (Lindenberg et al., 1985). In this mechanism of implantation, trophoblast cells intrude between the uterine epithelial cells to open intercellular junctions (Lindenberg et al., 1989). At the same time, new junctions are formed between trophoblast cells and the epithelium. There is no evidence that trophoblast cells phagocytose endometrial cells as they penetrate the epithelial layer (Lindenberg et al., 1986).

The first observations of the implantation process in vivo are from histologic sections of blastocysts at day 7, after epithelial penetration has taken place (Hertig, 1945). By this time, the cytotrophoblasts at the embryonic pole have proliferated and differentiated into two distinct layers: the inner layer is composed of mononuclear cytotrophoblast cells that are covered by an overlying multinucleated syncytium (Figure 1-1B). Detailed ultrastructural evidence from sections of human placentas from early pregnancy until term suggest that this syncytium is formed by cytotrophoblast fusion (Boyd and Hamilton, 1966; Hormann et al., 1969; Kaufmann and Stegner, 1972). In vitro, mononuclear cytotrophoblast stem cells can also fuse to syncytialize, recapitulating the in vivo process. In these cultures, cytotrophoblasts first aggregate and express desmoplakin, characteristic

of desmosome formation, and epithelial (E) -cadherin at points of cell-cell contact. Both of these markers subsequently disappear with cytotrophoblast fusion, along with an associated disintegration of the intercellular membranes (Contractor et al., 1977; Coutifaris et al., 1991; Douglas and King, 1990). Additional studies have shown that ^{3}H-thymidine is incorporated only into cytotrophoblast nuclei, and not syncytium (Kim and Benirschke, 1971; Weinberg et al., 1970). Thus, it is generally accepted that, because the syncytium cannot undergo mitosis to regenerate, the cytotrophoblast stem cells continuously proliferate then fuse to syncytialize (Benirschke and Kaufmann, 1995; Boyd and Hamilton, 1966).

The syncytium at the implantation pole in vivo subsequently displaces endometrial cells as the blastocyst embeds itself into the stroma (Boyd, 1970; Hamilton and Boyd, 1960; Hertig, 1949; Hertig, 1945). During this time, the endometrium begins to undergo a decidualization reaction in response to both mechanical and hormonal stimulation. Decidualization begins around the spiral arteries between days 6 and 8, then spreads throughout the uterus (Benirschke and Kaufmann, 1995). This process is primarily characterized by the differentiation of endometrial stromal cells (Enders and Schlafke, 1969; Spornitz, 1992). During their transformation into decidual cells, endometrial stromal cells become laden with lipid and glycogen and eventually take on a distinct polyhedral shape (Kaiser, 1960). Other histologic changes also take place, including the erosion of endometrial glands as the syncytium invades glandular epithelium (Genbacev, O., personal communication).

In the partially implanted 8 day embryo, cytotrophoblasts continue to surround the developing blastocyst. At this stage, the amniotic cavity begins to form between the embryoblast and the trophoblast at the embryonic pole (Hertig, 1941; Figure 1-1C). This space enlarges as implantation proceeds while amnioblasts delaminate from the overlying

cytotrophoblasts. The amnioblasts organize to enclose the cavity, forming the amniotic membrane. Two layers of trophoblast now separate the developing amniotic cavity from maternal endometrial tissue. These cytotrophoblast and syncytium layers comprise the primitive chorionic plate.

Concurrently, the embryoblast differentiates to form a bilaminar plate of cells called the embryonic disc, composed of two morphologically distinct cell layers: the epiblast, apposed the developing amniotic cavity, and the hypoblast, adjacent to the blastocyst cavity. Cells migrate from the hypoblast to enclose the exocoelomic cavity, which together form the primary yolk sac. During the second and third weeks of development, the yolk sac plays the important role of transferring nutrients to the developing embryo, prior to the establishment of a mature placental structure.

By day 10, the syncytium at the embryonic pole has enlarged and developed finger-like projections extending into the decidualizing endometrium (Boyd, 1970; Hertig et al., 1956; Figure 1-2A). Spaces called lacunae begin to form within the syncytial mass, giving the implantation site a spongy appearance. Individual lacunae are separated by columnar structures known as trabeculi, which are composed of a multinucleated syncytium that does not appear polarized at this stage. Because true syncytiotrophoblasts have not yet formed, it is inappropriate to use this term to describe the previllous implantation site. As implantation proceeds, the overlying syncytium, with its associated lacunae, extends from the embryonic pole to cover the entire surface of the conceptus.

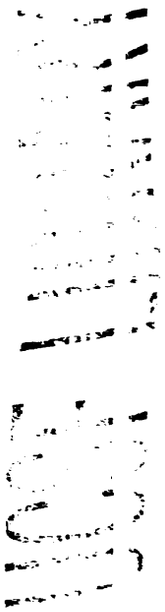
The blastocyst is completely embedded within the endometrium by day 12, and the uterine epithelium grows over the implantation site to repair the wound made by invading trophoblast cells. The fully implanted embryo can now be visualized as a small, raised dot on the lumenal surface of endometrial tissue (Hertig, 1941); O. Genbachev, personal

communication). At the same time in development, cytotrophoblasts begin to proliferate and extend from the chorionic plate, through the trabeculi, toward the decidualizing endometrium (Figure 1-2B). The cytotrophoblasts soon break through the syncytium, forming columns of cells that invade the decidua. At the end of the second week, they have formed an irregular layer surrounding the conceptus that is known as the cytotrophoblastic shell (Figure 1-2C). At the periphery of the cytotrophoblastic shell, some syncytial bodies can be seen within the decidua. It is unlikely that these trophoblast giant cells are the result of fragmentation of the syncytium, but are rather the result of the fusion of invasive cytotrophoblasts (Pijnenborg et al., 1981). Others believe that they are remnants of the multinucleated syncytium formed during implantation (O. Genbacev, personal communication).

The very rapid proliferation of trophoblast cells is an important feature of the second week of development. In this early stage of pregnancy, these extraembryonic cells expand tremendously relative to the minute size of the embryo. Because the yolk sac only provides nutrients until the third week, the trophoblast cells must quickly establish a mature placental structure with nutrient transport function to meet the embryo's growing demands. It is also remarkable to note the depth to which fetal trophoblasts have penetrated the uterine wall so soon after implantation. In such a short time, these aggressively invasive cells have populated the decidua, disrupting uterine glands and capillaries. With this behavior, trophoblasts display may of the characteristics of metastatic tumors. However, unlike the unrestricted growth of abnormal tumor cells, trophoblast invasion and differentiation in normal placentation is carefully regulated both temporally and spatially.

Placental Histology: the Villous Stage (two through six weeks)

Chorionic villi form during the second week of pregnancy, establishing the mature anatomy of the functional placenta. Histological sections of the conceptus at day 13 of development reveal that the lacunae are in communication (Figure 1-2C). The resulting large area, known as the intervillous space, now contains maternal erythrocytes (Boyd, 1970). Cytotrophoblasts have proliferated to fill the early trabeculi, thus forming structures known as primary chorionic villi. These villi are composed of a cytotrophoblastic core which is covered by a continuous layer of syncytium which essentially lines the intervillous space. Except during the early stages of implantation, the fusion of neighboring cytotrophoblasts to form syncytium has never been observed in vivo (Benirschke and Kaufmann, 1995). As shown in villous explant cultures, the syncytial mass most likely increases by the fusion of villous cytotrophoblasts with overlying syncytium (Castellucci et al., 1990). The apparent syncytialization seen in some cultures of isolated cytotrophoblast cells may be due to the presence of contaminating syncytial fragments.



At this stage, the multinucleated cells appear to be polarized, marking their differentiation into true syncytiotrophoblasts (Vanderpuye and Smith, 1987). The syncytiotrophoblast layer, anchored to the underlying cytotrophoblasts, is characterized by a highly convoluted apical plasma membrane, or brush border. Syncytiotrophoblasts are the only cell type in the body with branched microvilli on their apical surface (Boyd et al., 1968; Truman and Ford, 1984). The syncytiotrophoblast brush border is important for maximizing the surface area in contact with maternal blood in the intervillous space. The location and shape of syncytiotrophoblasts are ideally suited for their primary function, namely mediating nutrient, waste and gas exchange for the developing fetus.

Mesenchyme appears within the core of primary villi around day 15, thus forming secondary chorionic villi (Figure 1-2C). These undifferentiated stromal cells are the prevailing cell type until the end of the second month (Martinoli et al., 1984). Later in

pregnancy, mesenchymal cells are found in newly formed, immature villi. They are enmeshed in a loose network of reticular and collagen fibrils. This connective tissue is contained within a basement membrane upon which a monolayer of cytotrophoblasts, or Langhan's cells, is anchored. As in the primary villi, a continuous layer of syncytium lies above the cytotrophoblasts, covering the entire villous surface.

Around day 18, fetal macrophages are first seen within the stromal core (Figure 1-3B). These Hofbauer cells were first comprehensively described in normal villi at the beginning of the twentieth century (Hofbauer, 1903). Prior to establishment of the fetal circulation, it is thought that Hofbauer cells originate from mesenchymal cells in the villous core (Kaufmann et al., 1977). After the appearance of hematopoietic stem cells at around day 28, Hofbauer cells may differentiate from fetal monocytes, as other tissue macrophages do (Castellucci et al., 1987; Demir et al., 1989). Hofbauer cells share a number of characteristics with adult bone marrow-derived cells, although their precise function in the placenta has not been defined. For example, Hofbauer cells express class II major histocompatibility (MHC) molecules, those expressed by antigen presenting cells. It is thought that Hofbauer cells may be important for initiating a fetal response to placental infection by presenting antigens to fetal lymphocytes during pregnancy. Many additional immunological aspects of these cells have been extensively studied and are reviewed in (Benirschke and Kaufmann, 1995).

Other mesenchymal cells in the villous core differentiate to form hemangioblastic progenitor cells at around day 21-22 (Demir et al., 1989). These cells will give rise to primitive fetal capillaries in the following week of development, and by day 28, developing blood cells appear in the capillary lumens. The appearance of embryonic blood vessels within the mesenchymal core marks the formation of tertiary villi (Figure 1-3). By the end of the third week the chorionic villous capillaries are connected with the embryonic heart via vessels in

the connecting stalk, or primitive umbilical cord (Moore and Persaud, 1993). These mature chorionic villi comprise the functional unit of the human placenta, establishing the anatomical structures necessary for physiological exchange between the mother and developing embryo.

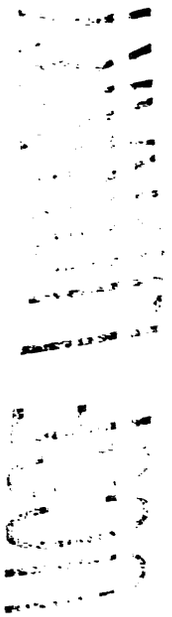
Histology of Mature Chorionic Villi (6 weeks to term)

To further maximize the area of exchange between maternal and fetal circulations, branches arise from the tertiary villi. This process begins by the local proliferation and fusion of cytotrophoblasts to form sprouts on the villous surface. These sprouts correspond structurally to immature primary villi, composed of a cytotrophoblast core surrounded by a layer of syncytiotrophoblast. The sprouts continue to grow and are soon invaded by mesenchyme, and later, by fetal capillaries. The result is formation of additional tertiary villi by a process analogous to the initial development of chorionic villi in the first steps of placentation. All tertiary villi further differentiate to form structurally and functionally distinct villous types (Figure 1-4A).

Of the two main categories of villi, floating chorionic villi are freely suspended within the intervillous space. These villi contain fetal capillaries within their stromal core and are surrounded by a pool of maternal blood in the intervillous space (Figure 1-4A). Their structure and location are ideal as the site of exchange between the fetal and maternal circulations. The basic histological architecture of mature floating villi persists throughout pregnancy, although some minor changes are seen with increasing placental age.

Until about 20 weeks gestation, four complete layers ensure the separation of maternal blood in the intervillous space, and fetal blood within the core of tertiary floating villi. Substances in maternal blood must pass syncytiotrophoblast, cytotrophoblast, stromal

connective tissue, and fetal capillary endothelium, respectively, as they are transported into the fetal circulation. Cytotrophoblasts fuse to form the overlying syncytium, and their rate of fusion is greater than that of proliferation. As a result, cytotrophoblasts are gradually depleted such that, after 20 weeks, their layer is incomplete (Moore and Persaud, 1993). At term, villous cytotrophoblasts, or Langhan's cells, appear as isolated cells surrounding the villous stromal core (Kaufmann, 1972). Although the cell layer is no longer continuous, the trophoblast basement membrane remains intact until term. In addition, the syncytiotrophoblasts continue to form a tight barrier covering the entire surface of the chorionic villi, in direct contact with maternal blood.



At the distal tips of some floating villi, cytotrophoblast stem cells do not fuse to form overlying syncytiotrophoblast, but rather proliferate to form aggregates of mononuclear cytotrophoblasts, known as cell islands. The precise function of these structures remains unknown (Benirschke and Kaufmann, 1995). Perhaps cell islands are early primary villous branches into which mesenchyme has not yet invaded. Others have suggested that floating villi containing cell islands are precursors of the second type of chorionic villi: anchoring villi.

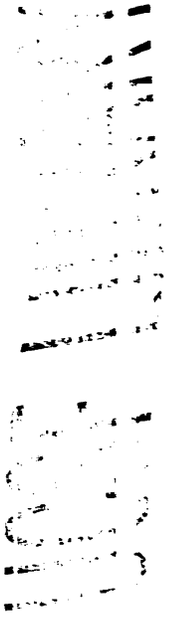
In contrast to floating villi, anchoring villi do not float freely in the intervillous space, but are directly attached to maternal tissue (Figures 1-4A, B). At the distal tips of anchoring villi, cytotrophoblasts form aggregates known as cell columns. Although these aggregates are similar to freely floating cell islands, columns are distinct in that they make contact with the uterine wall. At points of contact with maternal tissue, cytotrophoblasts differentiate to become invasive cells, migrating through the decidua. Interestingly, when floating villous explants containing cell islands are provided an extracellular matrix support in vitro, cytotrophoblasts within the islands also become invasive (Genbacev, O., personal communication). This observation lends support to the hypothesis that cytotrophoblastic

islands are analogous to, but topographically distinct from, the cell columns in anchoring villi.

In a process known as interstitial invasion, cytotrophoblasts from cell columns migrate deep into the uterine wall. These invasive extravillous cytotrophoblasts mix extensively with maternal decidual cells, thereby attaching the developing placenta to the uterus. Endometrial decidualization continues when, in the 6th week of pregnancy, the uterus is infiltrated with maternal leukocytes (Benirschke and Kaufmann, 1995). At this time, invasive cytotrophoblasts begin to come into contact with large numbers of maternal bone marrow-derived cells. These cell types include macrophages, T lymphocytes, granulocytes and large granular lymphocytes (LGL; (Bulmer and Johnson, 1984; Kaufmann, 1972; King and Loke, 1991; Starkey et al., 1988). Their precise role in the pregnant uterus is undefined, and has been the subject of considerable research. Although fetal extravillous cytotrophoblasts are allogeneic with respect to the mother, maternal immune cells do not appear to impede their invasion of the uterine wall. This topic will be discussed in greater detail in Chapter 2.

In their final stage of differentiation, cytotrophoblasts that emanate from columns also invade the tunica media of maternal spiral arteries, but not veins (Hamilton and Boyd, 1966; Pijnenborg et al., 1980; Figure 1-4B). This endovascular invasion results in the degeneration of the vascular internal elastic lamina and denudement of smooth muscle and elastin (Boyd, 1970). The arterial wall is then replaced with hyalin and fibrin, forming wide, patent vessels (Brosens et al., 1967; De Wolf et al., 1973). These changes reduce the vascular resistance to allow for maximal blood flow to the intervillous space. Cytotrophoblast interstitial and endovascular invasion in the first trimester thus serves to anchor the developing placenta to the uterus and to supply the growing embryo with maternal arterial blood, respectively.

The initial wave of cytotrophoblast invasion during the first trimester establishes the architecture of the maternal-fetal interface and begins to modify maternal spiral arteries. As pregnancy progresses, cytotrophoblasts continue to migrate until they reach the first third of the myometrium. By term, cytotrophoblasts can be seen in the inner media of myometrial segments of maternal spiral arteries (De Wolf et al., 1973; Sheppard and Bonnar, 1974). Invasive cytotrophoblasts have also replaced the endothelium in distal portions of these vessels (Benirschke and Kaufmann, 1995). These morphologic changes serve to augment the flow of arterial blood into the intervillous space. Through the differentiation of invasive cytotrophoblasts, anchoring villi function to attach the placenta to the uterus and access a supply of maternal blood for the fetus.



In summary, anchoring and floating chorionic villi are unique structures, both of which are important components of the human placenta. Together, they attach the placenta to the uterine wall, fill the intervillous space with arterial blood, and perform physiologic exchange between the mother and her fetus. These are the fundamental functions of the placenta, and are critical for the maintenance of normal pregnancy.

Molecular Aspects of Cytotrophoblast Differentiation

Cytotrophoblast differentiation along the invasive pathway is a complex process requiring the regulated expression of several classes of molecules. These include both cell-cell and cell-extracellular matrix (ECM) adhesion molecules as well as proteinases. With regards to cell-cell interactions, cytotrophoblast stem cells in the chorionic villous express E-cadherin which mediates homotypic adhesive interactions (Damsky et al., 1993). Villous cytotrophoblasts also express the α6 and ß4 integrin cell-ECM adhesion molecule subunits. The α6ß4 integrin dimer is expressed at the basal plasma membrane in many epithelia

(Sonnenberg et al., 1990) and probably represents a laminin (Ln) receptor (Damsky et al., 1992). Cytotrophoblast stem cells are adherent to their own basement membrane, surrounding the villous stromal core. Here, these cells secrete laminin, collagen IV and possibly merosin, a laminin-related protein (Damsky et al., 1992).

As they leave their basement membrane and begin to form columns, E-cadherin is downregulated and individual cytotrophoblasts begin to invade the decidua. Expression of α6ß4 is also downregulated and the cells turn on the α5ß1 fibronectin (Fn) receptor and begin to produce a Fn-rich matrix. Cytotrophoblasts within deeper portions of the uterine wall continue express α5ß1, but also upregulate the α1 subunit. Cytotrophoblast switching of adhesion molecule expression can be readily observed by immunohistochemistry of invasive cell columns in vivo (Damsky et al., 1992). In addition, purified cytotrophoblasts cultured on a basement membrane substrate recapitulate this behavior in vitro (Fisher et al., 1989). This model system has been used to show that the regulation of integrin expression is required for cytotrophoblast invasion (Damsky et al., 1994). Antibodies against either integrin α1ß1 or its ligands, collagen IV and Ln, inhibit cytotrophoblast invasive activity. Conversely, antibodies against α5ß1, upregulated as the cells leave their basement membrane in vivo, stimulate invasion. These results suggest that, while α1ß1-ligand interactions promote invasion, α5ß1-mediated adhesion is inhibitory.

During endovascular invasion, cytotrophoblasts replace the endothelium in maternal spiral arteries. In doing so, these cells continue to transform their adhesion molecule phenotype so as to mimic vascular endothelial cells. Specifically, endovascular cytotrophoblasts upregulate their expression of integrins αvß3 and α4ß1, in addition to the α4ß1 counter-receptor, vascular cell adhesion molecule-1 (VCAM-1), and the platelet-endothelial cell adhesion molecule-1 (PECAM-1) (Zhou et al., 1997). Expression of these cell-ECM adhesion molecules allows intra-arterial cytotrophoblasts to interact with the endothelial

basement membrane. Cytotrophoblasts also upregulate expression of the vascular-endothelial (VE)-cadherin. This adhesion molecule could mediate adhesion to maternal endothelial cells, and/or homotypic adhesive interactions among endovascular cytotrophoblasts. Function-perturbing antibodies to these adhesion molecules enhance invasiveness in the in vitro model of cytotrophoblast invasion, as well. Together, these results suggest that the correct modulation of adhesion molecule expression is critical for normal cytotrophoblast invasion.

In addition to adhesion molecules, proteinase secretion is also important for successful cytotrophoblast invasion of the uterine wall. To degrade the ECM through which they invade, cytotrophoblasts secrete members of both the plasminogen activators (PA) and matrix metalloproteinase (MMP) families. Although the PAs have limited ECM-degrading activity, they can activate MMPs, which are secreted as zymogens. Both the tissue-type and urokinase-type (t-PA and u-PA, respectively) can participate in a proteinase cascade in which plasmin ultimately cleaves procollagenase (Reich et al., 1988; Werb et al., 1977). While uPA is expressed by human cytotrophoblasts, inhibiting its activity does not prevent invasion in vitro (Librach et al., 1991; Queenan et al., 1987). However, it may indirectly affect matrix degredation and invasion through its activation of procollagenase. uPA may also be involved in regulating maternal blood flow to the placenta by preventing clot formation at the maternal-fetal interface. It has been suggested that plasma levels of uPA are significantly lower in pregnancies complicated by preeclampsia, a disease involving excess fibrin deposition in placental tissue (Lindoff and Astedt, 1994). Although uPA is not directly involved in cytotrophoblast invasion, blocking the activity of MMP-9, or the 92 kDa type IV collagenase/gelatinase, completely abrogates the cells' invasive capacity in vitro (Librach et al., 1991). MMP-9 secretion is downregulated in second and third trimester cells, paralleling the decrease in cytotrophoblast invasion as gestation progresses in vivo (Fisher et al., 1989; Librach et al., 1991).

The control of MMP activity in the uterine wall probably involves specific tissue inhibitors of metalloproteinases (TIMPs). It has been shown that the addition of either TIMP-1 or -2 to cultured cytotrophoblasts inhibits invasion (Librach et al., 1991). However, low levels of both of these inhibitors are constitutively expressed by cytotrophoblasts in vitro, suggesting that they probably do not play an autoregulatory role (K. Bass, et al., submitted). In contrast, production of the newest TIMP family member, TIMP-3, is significantly upregulated as cytotrophoblasts differentiate to an invasive phenotype. The parallel expression patterns of TIMP-3 and MMP-9 suggest that this proteinase-inhibitor pair is important for the modulation of cytotrophoblast invasive activity. In addition to the proposed autocrine regulation of cytotrophoblast invasion by TIMP-3, the decidua may be a source of paracrine-acting proteinase inhibitors. The mechanisms for the modulation of both adhesion molecule and proteinase expression are poorly understood. Because successful implantation is critical for normal pregnancy, these mechanisms undoubtedly include a complex milieu of regulatory factors at the maternal-fetal interface in vivo.

Gross Placental Anatomy: Development of the Definitive Discoidal Placenta

Through the second month of pregnancy, villi cover the entire surface of the chorionic sac, which contains the developing embryo (Figure 1-5A). The maternal tissue surrounding the embryo, known as the decidua, can be separated into three distinct regions according to its position relative to the implantation site. Immediately underlying the implantation site is the decidua basalis. This area contains deep projections of trophoblast cells and large numbers of maternal leukocytes. The portion overlying the embryo and facing the uterine lumen is the decidua capsularis. The maternal tissue opposite the implantation site is referred to as the decidua parietalis.

As the fetus grows beyond the 8th week, the chorionic villi of the decidua capsularis are compressed and their blood supply is reduced (Figure 1-5B). These villi begin to regress, resulting in formation of a relatively avascular bare membrane known as the chorion laeve by the third month of pregnancy (Boyd, 1970; Hamilton and Boyd, 1960). The amniotic sac, which contains the growing fetus within the chorionic sac, enlarges until it contacts and fuses with the chorion laeve around the 12th week (Boyd, 1970; Figure 1-5C). The resulting membrane is known as the amniochorionic membrane. The overlying decidua capsularis bulges into the uterine lumen and is stretched as the fetus grows within the uterine wall. This layer of decidua thins and eventually fuses with the amniochorionic membrane, then degenerates (Benirschke and Kaufmann, 1995). In the fourth month, the uterine lumen is completely filled and the amniochorionic membrane adheres to the decidua parietalis (Figure 1-5D). At the onset of labor, it is the amniochorionic membrane that ruptures to release amniotic fluid.

In contrast to atropic villi at the chorion laeve, those in contact with the decidua basalis undergo an extensive proliferation with a marked thickening and branching of stem villi (Figure 1-6). This part of the chorionic sac, known as the chorion frondosum, comprises the fetal portion of the definitive placenta. The underlying decidua basalis is its maternal component. These two tissues are separated by a gap known as the intervillous space. Fetal villi extend from the chorionic plate at the chorion frondosum into the intervillous space, toward the basal plate at the decidua basalis. Within the intervillous space, the chorionic villi branch extensively to form villous trees.

These structures are composed of several types of chorionic villi (Figure 1-7). Central stem villi are characterized by a condensed fibrous stroma containing arteries and veins, or arterioles and venules, whose media or adventitia is clearly visible by light microscopy. These truncus chorii are directly connected to the chorionic plate and divide up to four times

into short, thick branches called rami chorii of the first through fourth order. Additional generations of branching (2 to 30, with a mean of 10) are derived from each rami chorii, extending as slender branches into the periphery of the tree.

A subset of these slender ramuli chorii are anchoring villi, which are connected to the basal plate by a cytotrophoblast cell column. The remainder of ramuli chorii float freely within the intervillous space, and continue to branch into several intermediate villi. During the last trimester, terminal villi form as grape-like outgrowths from the intermediate villi. These villi contain fetal arteriocapillary venous networks that continue to proliferate after villous longitudinal growth has ceased, thus causing the villous tips to dilate.

The extensive branching of the chorionic villi serves to increase both the placental thickness and surface area. The placenta continues to grow until about the fifth month, when it covers approximately 15 to 30 percent of the uterine surface. The fully developed placenta is usually discoid in shape, and contains hundreds of chorionic villi, its basic functional unit. The total villous area measures 2.5 m^2 at the end of the second trimester, increasing to about 12 m^2 at term (Benirschke and Kaufmann, 1995). The actual area of physiologic exchange at term is greater by a factor of approximately 7.67, due to the increased surface area provided by the syncytiotrophoblast microvilli (Teasdale and Jean-Jacques, 1985).

Physiologic exchange takes place between fetal blood in the villous core and maternal blood in the intervillous space. This space is filled with blood from spiral arteries in the decidua basalis, and sealed by the overlying chorionic plate (Figure 1-6). The maternal blood pressure within the spiral arteries is significantly higher than in the intervillous space. Thus, blood is released in pulsatile fountains called Borell's jets, as they were first described in human pregnancy by Borell et al (Borell et al., 1958). Blood percolates slowly among the villi, then is drained by endometrial veins, resulting in a constant

circulation of maternal blood through the placenta. At term, the intervillous space contains about 150 ml of blood that is replenished three or four times a minute (Metcalfe et al., 1955; Moore and Persaud, 1993).

During abortion or delivery at parturition, the placenta separates from the uterine wall through the decidua basalis, severing the spiral arteries. Contractions of the uterine myometrium then constrict these arteries to control excessive bleeding. The result is the expulsion of tissues including the junction of fetal chorionic villi and maternal decidua, or the placental bed. Biopsies of this region can also be taken directly from the uterine wall at the time of cesarean section, immediately following delivery of the placenta (Zhou et al., 1993). Tissue sections of the mature placental bed are useful for observing the histologic architecture of the feto-maternal interface. Additionally, the expression of different classes of molecules can be studied using immunohistochemistry or in situ hybridization techniques. Cytokeratin has been shown to be a useful marker for all trophoblastic cells, including villous and extravillous populations (Fisher et al., 1989). Most recently, methods have been developed for isolating distinct populations of cells from placental tissue (Fisher et al., 1989; Kliman et al., 1986). This allows researchers to perform in vitro studies to gain a better understanding of the events that take place during the morphogenesis of this fascinating organ.

Pathological Placentation

Because correct formation of the placenta is critical for normal fetal development, errors in placentation can have severe negative effects of pregnancy outcome. Placental defects can arise at any time during development, beginning with blastocyst apposition to the uterine wall. In normal pregnancy, the blastocyst attaches to the endometrium via the embryonic pole. Rarely, the blastocyst is rotated such that endometrial adhesion is not at the

embryonic pole. This results in the relatively harmless insertion of the umbilical cord at the margins, rather than near or at the center of the placenta (Benirschke and Kaufmann, 1995). Marginal cord insertion, known as Battledore placenta, occurs in nearly 7% of term placentas (Benirschke and Kaufmann, 1995). The most extreme example of this defect is in velamentous insertion. In this case, the cord inserts on the amniochorionic membrane and is more vulnerable to trauma and disruption.

In addition to correct blastocyst orientation, the location of implantation is important for correct placental development. Most embryos implant and form a placenta in the upper, posterior uterine wall, or fundus (Hertig and Rock, 1973). When implantation is low in the uterus and the placenta grows over the internal cervical os, the result is a dangerous condition known as placenta previa (Mabie, 1992). In this case, the fetus cannot be delivered first, and there is a risk of severe maternal and fetal hemorrhage during pregnancy or labor. The blastocyst can also incorrectly implant in ectopic sites, such as the fallopian tube or abdominal cavity. Virtually all tubal pregnancies terminate prematurely due to rupture and overdistention of the fallopian tube. In contrast, although their incidence is quite rare, term abdominal pregnancies have been described (Martin et al., 1988). The success of these pregnancies suggests that the placenta can support fetal development to term in the absence of decidualized uterine tissue.

Intrauterine pregnancies are also sometimes associated with decidual defects, resulting in abnormal placentation. When the decidua is absent at sites of blastocyst attachment, the developing villi become anchored to muscle fibers, with villous tissue in direct contact with the myometrium. This decidual deficiency prevents normal separation of the placenta at delivery, and portions or all of the placenta may be retained, necessitating surgical removal. This disorder is known as placenta accreta and can be distinguished from increta and percreta on the basis of the depth to which chorionic villi penetrate the uterus. In placenta

increta, the entire myometrium is invaded, whereas with percretas, villi penetrate the entire wall and the uterus ruptures. By definition, ectopic pregnancies are characterized by placenta accreta due to the absence of decidual tissue at extrauterine sites.

Defects in the depth of uterine penetration can also result from either poorly or overly invasive cytotrophoblast cells. Normally, extravillous cytotrophoblasts form columns emanating from the tips of anchoring villi, and migrate into the decidua (Figure 1-4A). These invasive cells attach the placenta to the uterus and also remodel maternal spiral arteries to supply blood to the intervillous space. When cytotrophoblast invasion is abnormally shallow, this blood supply is reduced, resulting in a decreased nutrient source for the developing fetus. Insufficient cytotrophoblast invasion has been associated with approximately half the cases of intrauterine growth retardation (IUGR) (Gerretsen et al., 1981).

Shallow invasion is also an anatomical feature of preeclampsia, a syndrome involving fetal IUGR and associated maternal morbidity (Friedman et al., 1991). Preeclampsia is a disease affecting 7 to 10 percent of all pregnancies, with a higher incidence in primigravidas and multifetal gestations, or those with increased placental mass (reviewed in Friedman et al., 1991; Lim and Friedman, 1993; Roberts et al., 1993). It is characterized by a systemic pathophysiology including maternal hypertension, proteinurea, and edema developing in the second trimester. These symptoms are secondary to reduced organ perfusion caused by an increased vascular sensitivity to pressors and activation of the coagulation cascade (Lindheimer and Katz, 1989; Pritchard et al., 1976). Inappropriate fibrin deposition results in the formation of microthrombi that occlude vessels throughout the maternal vasculature, including uterine spiral arteries (Redman, 1990; Vassali et al., 1963). Without prophylactic therapy, patients can ultimately develop eclampsia, manifested by central nervous system (CNS) involvement with seizure activity (Lopez-

Llera, 1992). While its cause remains undefined, the only known cure for preeclampsia is delivery of the placenta, implicating this organ in the etiology of the disease (Redman, 1991). In fact, preeclampsia has been described in patients with hydatidiform mole, in the absence of a fetus (Chun et al., 1964).

The histology of the preeclamptic basal plate reveals abnormally shallow placentation with an absence of invasive cytotrophoblasts in the myometrium. This may be the result of the failure of differentiating cytotrophoblasts to downregulate expression of integrins α6ß4, those normally expressed only by villous cytotrophoblasts (Zhou et al., 1993). In addition, preeclamptic cells do not upregulate the α1ß1 complex, unlike the invasive cytotrophoblasts from normal placentas. Data from in vitro studies with purified cytotrophoblasts demonstrates that α1ß1-ligand interactions promote invasion (Damsky et al., 1994). These observations from preeclamptic placentas confirm that the correct expression of integrin molecules is required for normal interstitial invasion in vivo.

Endovascular invasion is also compromised in preeclampsia. Evidence suggests that abnormal adhesion molecule expression may result in the inability of preeclamptic cytotrophoblasts to mimic a vascular adhesion phenotype (Zhou et al., 1997). Unlike cells from normal placentas, preeclamptic cytotrophoblasts fail to upregulate αvß3, α1ß1, VE-cadherin, VCAM-1 and PECAM-1. In vitro analyses suggest that expression of these adhesion molecules by normal cytotrophoblasts stimulates invasion (Zhou et al., 1997). In preeclampsia, cytotrophoblasts are limited to superficial portions of the spiral arteries. This results in a significant reduction in the arterial blood flow to the intervillous space (Brosens et al., 1972; Gerretsen et al., 1981; Lunell et al., 1982).

As a consequence of decreased arterial blood flow, cytotrophoblast stem cells remain in a relatively hypoxic environment. Exposure of purified cytotrophoblasts to a reduced

oxygen tension environment in vitro affects their progression through the cell cycle (Genbacev et al., 1996). Instead of terminally differentiating into invasive cells, hypoxic cytotrophoblasts proliferate as evidenced by an upregulation of DNA synthesis. Increased cytotrophoblast proliferation is also evident in villous explants cultured under hypoxic conditions (Burton, 89). Hypoxic cytotrophoblasts also fail to upregulate integrins $\alpha 1\beta 1$, as is observed in preeclampsia in vivo. The net result is a reduction in the invasive capacity of cells cultured under hypoxic conditions. These findings suggest that the low oxygen tension environment in preeclampsia can inhibit cytotrophoblast differentiation along the invasive pathway.

The anatomy of preeclamptic placentas suggests that a reduction in oxygen tension also affects cytotrophoblasts in vivo (Benirschke and Kaufmann, 1995). Increased cytotrophoblast stem cell proliferation results in villous maldevelopment in these placentas. Like cytotrophoblast stem cells, fetal capillary growth is also stimulated by prolonged hypoxia, resulting in multiply branched and highly coiled capillary loops. Subsequently, the villi of preeclamptic placentas are highly branched with knobby, indented surfaces, representing areas of capillary protrusion into the villous wall. These structural modifications serve to increase the surface area for physiologic exchange between fetal and nutrient-poor maternal blood. It has been suggested that the poorly perfused preeclamptic placenta produces a factor that causes widespread endothelial cell damage, resulting in the observed systemic manifestations of the disease (Roberts et al., 1993).

In contrast to disorders resulting from deficient invasion, unrestricted proliferation of cyto- and syncytiotrophoblast cells can lead to gestational trophoblastic diseases. These comprise a spectrum of interrelated tumors that vary in their degree of invasiveness and metastatic potential (reviewed in Berkowitz and Goldstein, 1996). Hydatidiform moles are the least invasive and result from the growth of immature chorionic villi in the absence of

identifiable embryonic or fetal tissue. These villi are not vascularized, and form cystic swellings containing hyperplastic trophoblast cells. Moles are androgenetic, with all chromosomes paternally derived (Kajii and Ohama, 1977). This karyotype can often result from the fertilization of an "empty" egg, or one containing no maternal chromosomes, with a subsequent duplication of the haploid spermatozoal complement. Because 46,YY is a lethal condition, the vast majority of moles are 46,XX, formed by fertilization by one X-bearing sperm. 5-10% of moles are androgenetic yet heterozygous, the result of fusion of two male pronuclei after dispermic fertilization, with karyotypes of either 46,XX or 46,XY (Ford et al., 1986). In addition, mixed populations of cells with different karyotypes and different ploidies occur in rare mosaic moles (reviewed in Benirschke and Kaufmann, 1995).

After evacuation of a molar pregnancy, two to four percent of patients develop persistent tumors known as choriocarcinomas. These tumors can also follow normal delivery or abortion and are characterized by sheets of undifferentiated cytotrophoblast and syncytiotrophoblast without chorionic villi. Although choriocarcinomas often metastasize widely, they are extremely sensitive to chemotherapy and are curable in most cases. Placental site trophoblastic tumor (PSTT) is a very rare variant of choriocarcinoma that consist mostly of differentiated intermediate, invasive cytotrophoblasts (Denny et al., 1995). Unlike choriocarcinoma, these tumors tend to remain within the uterus, extensively invading the myometrium and vessel walls. This behavior appears to simulate normal uterine invasion by extravillous cytotrophoblasts. Unfortunately, PSTTs are relatively insensitive to chemotherapy thus hysterectomy is the optimal treatment for these tumors.

Placental Physiology: Comprehensive Functions

As a physical interface between the mother and fetus, the placenta forms an important structural connection while performing a variety of diverse physiologic functions. Starting early in pregnancy, the placenta acts as an endocrine organ whose products are secreted into the maternal circulation. Human chorionic gonadotropin (hCG) is produced by invasive cytotrophoblasts as well as by syncytiotrophoblast in the first trimester (Gosseye and Fox, 1984). In fact, hCG can be found in maternal urine as early as the second week of development, and its detection is commonly used for diagnosing pregnancy (Chard, 1992). Both syncytial and extravillous cytotrophoblast hCG protein and mRNA expression decreases over time, dropping to levels undetectable by immunohistochemistry at term (Hoshina et al., 1982; Sabet et al., 1989). In contrast, human placental lactogen (hPL) is produced by both invasive cytotrophoblasts and syncytium throughout pregnancy (Gosseye and Fox, 1984). Neither of these placental protein hormones have been detected in villous cytotrophoblast stem cells (Daya and Sabet, 1991). Syncytiotrophoblasts also synthesize several steroid hormones including progesterone, which is critical for the maintenance of pregnancy, and estrogens (reviewed in Strauss et al., 1996). Essential fetal nutrients such as glycogen, cholesterol, and fatty acids are also produced by the placenta (Moore and Persaud, 1993).

In addition to these important endocrine and metabolic functions, the placenta mediates the transport of substances from maternal blood in the intervillous space to the fetal circulation. Materials cross layers of the chorionic villi by either simple diffusion, facilitated diffusion, active transport, or pinocytosis (Moore and Persaud, 1993). Oxygen and carbon dioxide move by simple diffusion between maternal and fetal blood. Gas exchange across the placenta is as efficient as in the lung, consequently fetal hypoxia results primarily from limited uterine blood flow (Benirschke and Kaufmann, 1995). Water is also freely transported by simple diffusion, moving from areas of high concentration to those of low concentration until equilibrium is reached. Vitamins and glucose in the maternal circulation

cross the placenta, although cholesterol, triglycerides, and phospholipids do not. While these nutrients are transported into the fetal circulation, waste products including urea, uric acid, and bilirubin are released into intervillous blood to be cleared by the mother.

Although the fetus does not have the capacity to produce antibodies, the transplacental transfer of maternal antibodies can also provide passive immunity against a variety of antigens. Alpha and beta globulins are transported in very small amounts, although the gamma globulins, specifically the IgG class, are readily transported to the fetus during normal pregnancy. However, the mechanisms of transplacental transfer of maternal IgG have not been definitively identified (reviewed in Kristoffersen, 1996). The IgG transporter, Fc receptor (FcR) -III, and annexin II, with low affinity FcR activity, are present on the syncytiotrophoblast apical cell membrane. Both molecules face the maternal blood in the intervillous space and either one or both may mediate the binding and transfer of maternal IgG. In addition to these, the human homologue of the murine neonatal FcR (FcRn), essential for maternal IgG transport in the mouse, has been defined (Leach et al., 1996; Story et al., 1994). hFcRn co-localizes with intracellular IgG in syncytiotrophoblast endocytotic granules, and is possibly involved in the protected transfer of IgG across the syncytium layer. These molecules are not expressed in fetal capillary endothelia within the villous stromal core. Therefore, the mechanism of maternal IgG transport across this second cellular barrier into the fetal circulation remains unknown.

Although placental transport function is critical for normal fetal development, it is not a completely selective process, allowing a variety of harmful substances to enter the fetal circulation. For example, it is widely known that most drugs ingested by the mother are readily delivered to the fetus with dangerous consequences. Maternal alcohol consumption during pregnancy results in fetal alcohol syndrome (FAS), characterized by pre- and postnatal growth retardation, central nervous system dysfunction, and craniofacial

dysmorphology (Appelbaum, 1995). The use of cocaine and crack by pregnant mothers is also deleterious for normal fetal development, often resulting in growth retardation and intrauterine drug addiction (Chasnoff et al., 1985; Chasnoff et al., 1989). Cocaine also affects placental function, frequently causing preterm delivery and abruptio placentas. Much of these effects may be mediated through cocaine's known hypertensive and vasoconstrictive activities (Woods et al., 1987). Maternal and fetal effects of cigarette smoking include an increased incidence of spontaneous abortions in the first trimester, and increased preterm delivery rates with decreased birth weights at term (reviewed in landers). Nicotine readily traverses the syncytiotrophoblast layer to gain access to the fetal circulation and may have vasoconstrictive effects on the umbilical artery. In addition, chorionic villi of smoking mothers show a marked reduction in invasive cytotrophoblast columns, as do normal villi exposed to nicotine in culture (Genbacev, et al., 1995). Nicotine also inhibits the invasive activity of isolated first trimester cytotrophoblasts, suggesting that defects in placental attachment may contribute to the effects of maternal smoking.

Even drugs that are beneficial to the mother, such as antimicrobial therapy, cross the placenta and can expose the fetus to risk (reviewed in Garland and O'Reilly, 1995). Some classes of antibiotics are safe throughout pregnancy (e.g. beta-lactams) while others are completely contraindicated (e.g. tetracyclines) (reviewed in Korzeniowski, 1995). Still others are to be avoided only at certain times in pregnancy (e.g. sulphas should not be administered in the third trimester). The risks of systemic antifungal treatment during pregnancy are unclear, however oral fluconazole therapy is contraindicated in pregnancy. Although it has been associated with severe congenital anomalies in few cases (Pursley et al., 1996), very few adverse affects were observed in a large group of pregnant women using oral fluconazole to treat vaginal candidiasis (Inman et al., 1994). Steroid hormones in the maternal circulation can also readily traverse the placenta. For example, virulizing

steroids injested by the mother can result in masculinization of a chromosomally female (XX) fetus (Blyth and Churchill, 1996).

A number of microorganisms can also breech the placental barrier to enter the fetal blood (see Altshuler, 1984 for a comprehensive review of placental infections). The chorionic villi and amniotic sac can become infected by organisms that ascend through an open endocervical canal. The resulting chorioamnionitis is an acute inflammatory reaction that can often cause preterm delivery (Hillier et al., 1988). Placental bacterial infection and subsequent fetal transmission have been described in tuberculosis (Abughali et al., 1994), listeriosis (Topalovski et al., 1993), and syphilis (Evans and Frenkel, 1994). With regards to parasitic infections, transplacental transmission of toxoplasmosis is quite common and can cause severe crippling disease in the fetus (Matsui, 1994). In contrast, congenital malaria infection has been described only rarely, although it is one of the most common parasitic diseases in the world (Hulbert, 1992). Vaginal candida infection is an exceedingly common fungal infection during pregnancy, but rarely passes the cervical mucous plug to infect the placental membranes. Among other infections caused by fungi, coccidioidomycosis is a rare disease in which uterine involvement remains confined to the placenta. Neonatal infection has been observed and is thought to occur by inhalation of contaminated maternal material during delivery (Peterson et al., 1993).

Among viral infections that can cross the placenta, those caused by cytomegalovirus (CMV) are quite common, with an estimated 3,000 to 4,000 affected infants born in the US each year (Yow, 1989). Congenital CMV infection can result in hearing loss, blindness, mental retardation, and in the most severe cases, fetal death. Although neonates can become infected with herpes simplex virus (HSV) type 2 during contact with maternal genital lesions during delivery, transplacental HSV infection is rare (Baldwin and Whitley, 1989). This may be because of the protective nature of transplacentally transferred

maternal antibodies against HSV. High titers of human immunodeficiency virus (HIV) DNA within 48 h of birth is evidence that vertical transmission of this virus occurs (Brandt et al., 1994). Although, little is known about the timing and mechanism of tranplacental fetal HIV infection (Douglas et al., 1991; Michie and Hyer, 1995). Studies suggesting placental cell (e.g., trophoblast, stromal and Hofbauer) infectibility with HIV have been controversial and difficult to reproduce (Amirhessami-Aghili and Spector, 1991; De Andreis et al., 1996; Greenspoon and Settlage, 1989). In addition, the viral mode of entry through the villous stroma and into the fetal circulation is unknown.

Neonatal infection also frequently occurs at birth, when the placental barrier is transiently broken, as well as in late pregnancy, as the aging placenta becomes leaky. During parturition (Lloyd et al., 1980) or abdominal trauma (Pearlman et al., 1990), mechanical stress may cause microscopic leaks to form in the placental circulation, resulting in a mixing of fetal and maternal blood. Significant transplacental hemorrhage can result in fetal anemia or even exsanguination and death in the most severe cases (Crane et al., 1993). Although the transfer of maternal cells into the fetal circulation is quite rare, occasional cases of this phenomenon have been documented (Page et al., 1981). More often, a break in the placental barrier can allow maternal exposure to fetal blood cells.

Fetal erythrocytes are most commonly identified in maternal blood by the well established Kleihauer-Betke method (Kleihauer et al., 1957). This technique involves the separation of erythrocytes on the basis of the differential solubility of fetal and adult forms of hemoglobin in acidic Ph. Several modifications have been made to improve upon this technique and are reviewed in Benirschke and Kaufmann (1995). The leakage of small amounts of Rh positive fetal blood into the circulation of an Rh negative mother can lead to maternal immunization against fetal Rh antigens. The transplacental transfer of the resulting maternal antibodies can then cause fetal hemolytic disease. If maternal

isoimmunization occurs early in gestation, the fetus is at risk as the pregnancy progresses and may require transfusions in utero through the umbilical vein (Grannum et al., 1988). In most cases, isoimmunization takes place at parturition, at which time the mother is administered anti-Rh immunoglobulin (RhoGam) prophylaxis to prevent the development of this disease in subsequent pregnancies (Mittendorf and Williams, 1991).

Other fetal cells are also frequently detected in maternal blood and tissues in normal pregnancy. The presence of syncytiotrophoblast in the maternal lung was described over a century ago (Schmorl, 1893; Schmorl, 1905). Transplacental transfer of fetal lymphocytes also occurs, and these cells can be detected in maternal blood or bone marrow for years post partum (Schroder, 1975). Using the polymerase chain reaction (PCR) to identify Y chromosome-specific sequences in the maternal peripheral blood, it was recently suggested that nucleated fetal cells regularly traverse the placental barrier (Holzgreve et al., 1990). Efforts are currently underway to enrich fetal cells from maternal blood to be used as a potential source for prenatal diagnosis (reviewed in Bianchi, 1995). These include both trophoblast and nucleated fetal red blood cells.

The placenta is a unique structure in that, during its limited lifetime, it concurrently displays characteristics of many individual organ systems. It is an endocrine organ, synthesizing a variety of hormones for release into the maternal circulation. As a respiratory organ, the placenta exchanges oxygen and carbon dioxide for the developing fetus. At the same time, the placenta also functions as a kidney, excreting metabolic waste products back into maternal blood, and as a liver, performing important metabolic and secretory functions. It closely resembles the gut in its ability to transport nutrients into the fetal circulation. However, the placenta is an imperfect barrier as many harmful substances are also transported to the fetus.

Comparative Mammalian Placentation

Although all viviparous vertebrates develop a placenta, this organ exhibits more diversity among the species than any other. The only architectural similarity all types of placentas share is the separation of maternal and fetal circulatory systems. The conditions of pregnancy differ vastly in different vertebrate species, including litter size, length of gestation, and fetal size. Therefore, one conserved structure cannot fulfill the same functional requirements for different animals. Human placentas are unique structurally from even closely related mammals in a number of ways, including overall shape, type of feto-maternal contact, barrier separating fetal and maternal blood, and placental bed histology. Because the comparative placentological literature is so vast, this summary will focus on differences between human and primate species, as well as rodents, as they are commonly used as laboratory models of normal and pathological human placentation. For a comprehensive review of this field, see the discussion on placental types in Benirschke and Kaufmann (Benirschke and Kaufmann, 1995).

Differences in placentation among various animal species begin at the earliest stage of development, namely uterine implantation. In pig and horse pregnancies, the attached blastocyst does not penetrate the uterine epithelium, resulting in formation of an epitheliochorial placenta. Rodent, human, and primate trophoblasts invade the uterine epithelium and stroma to form a hemochorial placenta, where fetal tissue is in contact with maternal blood. Among mechanisms used to disrupt the epithelium, displacement, fusion, and intrusive types of invasive implantation have been described (Benirschke and Kaufmann, 1995). Displacement, which is usually observed in rodents, involves the degeneration of the uterine epithelium after blastocyst attachment to its apical surface. The subsequent penetration of the blastocyst into the uterine stroma is mediated by the decidual cells, rather then by trophoblast penetration. In the fusion type of implantation, the apical

plasma membranes of the attaching trophoblast cells and uterine epithelial cells fuse to form a mixed syncytium. This maternal and fetal syncytium then penetrates the underlying uterine stroma in a process described in the rabbit. Lastly, the rhesus monkey and human display an intrusive type of implantation (Enders et al., 1983; Lindenberg et al., 1985). In these species, extensions of syncytiotrophoblast intrude into the intercellular space between epithelial cells, breaking their intercellular junctions and forming junctional complexes with the epithelium.

The human blastocyst continues to invade the uterine stroma after penetrating the epithelial cell layer. The result is an example of interstitial implantation, where the blastocyst is completely embedded within maternal tissue (Figures 1-2A and 1-5). In contrast, rhesus monkey and baboon blastocysts remain superficially attached to the uterine wall (Ramsey et al., 1976). As the embryos grow, they eventually reach the uterine wall opposite the original implantation site. The rhesus, but not the baboon, forms a second attachment here. To maximize the surface area of materno-fetal contact at the site(s) of attachment, the placenta forms as an interdigitation of both tissues.

Interdigitation extending over the entire surface of the chorionic sac, placenta diffusa, is seen in some lower primates such as the galago (Ramsey, 1982; Figure 1-8A). In other cases the interdigitation is so extensive that a limited area provides sufficient fetal-maternal contact. Some animals, including dogs and cats, develop a ring-like placenta surrounding the fetus like a girdle, known as a placenta zonaria (Figure 1-8B). The placentas of other animals, such as the rhesus monkey, form two distinct areas of contact to form a bidiscoid placenta (Figure 1-8C). In the discoidal placenta, a single disc-shaped region of contact provides the most highly concentrated area of feto-maternal interdigitation (Figure 1-8D). This structure, formed in normal human pregnancy, is also shared by rodents and the great apes (Benirschke and Kaufmann, 1995).

The type of interdigitation varies among different placentas, as well. In the folded placenta, the area of contact is characterized by folds of the chorion that fit into corresponding grooves in the uterine mucosa (Figure 1-9A). Because this shape provides only a small increase in surface area relative to a planar apposition, the corresponding region of feto-maternal contact may be quite large. In fact, this type of placentation is seen in some animals with a placenta diffusa, such as the pig. In the discoidal placenta, the limited area of feto-maternal contact requires that the interdigitations increase in complexity to provide a larger exchange surface area. An extensive branching of chorionic villi can be found in human placentas as well as those of most higher primates (Boyd, 1970). In this villous type of placentation, fetal chorionic villi are completely surrounded by maternal blood (Figure 1-9B). The most efficient type of interdigitation is seen in the labyrinthine placenta of rodents and some lower monkeys such as the tupia (Ramsey, 1982; Wynn, 1964). These placentas have a spongy appearance, with channels of either fetal capillaries or maternal blood directly penetrating trophoblastic tissue (Figure 1-9C).

While the type of exchange area varies widely among different species, each placenta maintains a complete separation of fetal and maternal blood. However, the cell layers that comprise the barrier between the two circulations are distinct in each case (Figure 1-10). In some animals, such as the dog, cat, and lower primate, tupaia, trophoblast invasion results in a direct apposition of these fetal cells with maternal endothelium (Benirschke and Kaufmann, 1995). In this endotheliochorial placenta, maternal blood is separated from the fetal circulation by maternal capillary endothelium, trophoblast, villous stromal tissue, and fetal endothelium (Figure 1-10A). With continued invasion and disruption of maternal vessel walls, trophoblast cells make direct contact with maternal blood in forming a hemochorial placenta. This type of placenta can be further subdivided on the basis of the number of trophoblast layers separating fetal and maternal blood (Enders, 1965; Wynn and

Davies, 1965). In the hemotrichorial placenta, seen in rat, mouse, and hamster pregnancies, substances in maternal blood cross layers of cytotrophoblast, syncytiotrophoblast, and cytotrophoblast again, before reaching the fetal circulation in the villous core (Figure 1-10B). First trimester human and rabbit placentas are hemodichorial. In this case, a monolayer of cytotrophoblasts is covered by syncytium, which, in turn, is in direct contact with maternal blood (Figure 1-10C). As the human placenta ages, the cytotrophoblast layer is depleted such that, by term, this placenta is of the hemomonochorial type, with only a single layer of syncytium separating maternal blood from the fetal capillaries (Figure 1-10D).

Significant histological differences also exist in the placental bed of each species. While invasive human cytotrophoblasts migrate through the first third of the uterine myometrium, murine trophoblasts are not found in this muscle layer (Damsky et al., 1993). Whether or not myometrial segments of uterine arteries are invaded by trophoblast cells in macaque and baboon pregnancies is unclear. Ramsey et al. concluded that trophoblast does not penetrate the endometrial-myometrial junction in these primate species (Ramsey et al., 1976). However, later observations suggest that trophoblasts invasion of spiral arteries in the myometrium does occur (Blankenship et al., 1993).

The significance of these anatomical differences is illustrated by the fact that preeclampsia, a disease of human pregnancy characterized by deficient cytotrophoblast myometrial and spiral artery invasion, is not naturally occurring in other animal species. Recently, a murine model for pregnancy-induced hypertension was developed that involves mating transgenic mice expressing components of the human renin-angiotensin system (Takimoto et al., 1996). Like the human disease, pregnant females experienced a transient elevation in blood pressure in late pregnancy that returned to normal levels after delivery. Although placental necrosis and edema were observed, it is difficult to compare this pathologic

histology to that seen in preeclampsia, as the morphologies of the implantation sites in these two species are distinct. In spite of this significant difference, these mice may be useful in providing some insight into the molecular mechanism of pregnancy-associated hypertension.

One unifying characteristic among viviparous animals is that fetal tissue is genetically dissimilar to the maternal tissue into which it implants. However, each animal must have evolved distinct mechanisms for maternal immune tolerance of the genetically foreign fetus. Whereas human cytotrophoblasts mingle extensively with decidual cells, a definitive boundary exists between fetal and maternal cells in mouse and related primate basal plates (Damsky et al., 1993; Ramsey, 1982). While up to 70% of the cellular makeup of the human decidua is bone-marrow derived, hematopoietic cells are largely excluded from the mouse and rat decidual zones (Bulmer and Sunderland, 1984; Tachi and Tachi, 1989). In this regard, the relationships between fetal and maternal cell types must be unique in each pregnancy. Another obvious difference is in the gestational length in each species: ranging from 21 days in the mouse to 20 weeks for rhesus monkeys and 9 months for a term human pregnancy. The extended length of maternal exposure to fetal antigens in human pregnancy should allow sufficient time for the generation of a deleterious immune response, however one is not mounted.

The unique qualities of human pregnancy immunology are exemplified by the fact that a homologue of the human class I MHC gene, human leukocyte antigen-G (HLA-G), has not been found in other animal species. HLA-G is expressed by extravillous cytotrophoblast cells and may be important in mediating protective immune interactions with decidual leukocytes in the uterine wall (McMaster et al., 1995; Pazmany et al., 1996). A novel murine MHC class I gene, blastocyst MHC, has been recently cloned and shown to be expressed at the blastocyst stage and in the placenta (Sipes et al., 1996). The authors

suggest that blastocyst MHC may be the mouse analog for HLA-G, but further characterization of its expression pattern is required before this can be concluded.

These significant differences between different species underscore the limitations of working with animal models of human pregnancy. While human placentas are readily available for in vitro research, animals are required for any in vivo experiments. Only the placentas of the great apes are directly comparable to their human counterparts, but these animals are not readily available for use in the laboratory. Thus, researchers are limited to working with animals whose placentas are quite different morphologically and functionally from those of humans. Although some common genetic elements exist, the placentas of each species are exposed to vastly different environments in utero. We must therefore be quite cautious in drawing functional conclusions about human pregnancies from observations made in animal systems.

Figure 1-1: Drawings illustrating blastocyst apposition and the early stages of implantation. A, Day six: the blastocyst is attached to the uterine epithelium via the trophoblast cells at the embryonic pole, immediately overlying the inner cell mass. B, Day seven: the cytotrophoblast at the embryonic pole have fused to form a syncytium, which penetrates the epithelium and begins to invade the uterine stroma. C, Day eight: the amniotic cavity begins to form between the cytotrophoblasts at the embryonic pole and the embryonic disc. (Modified from Moore and Persaud, 1993, with permission).

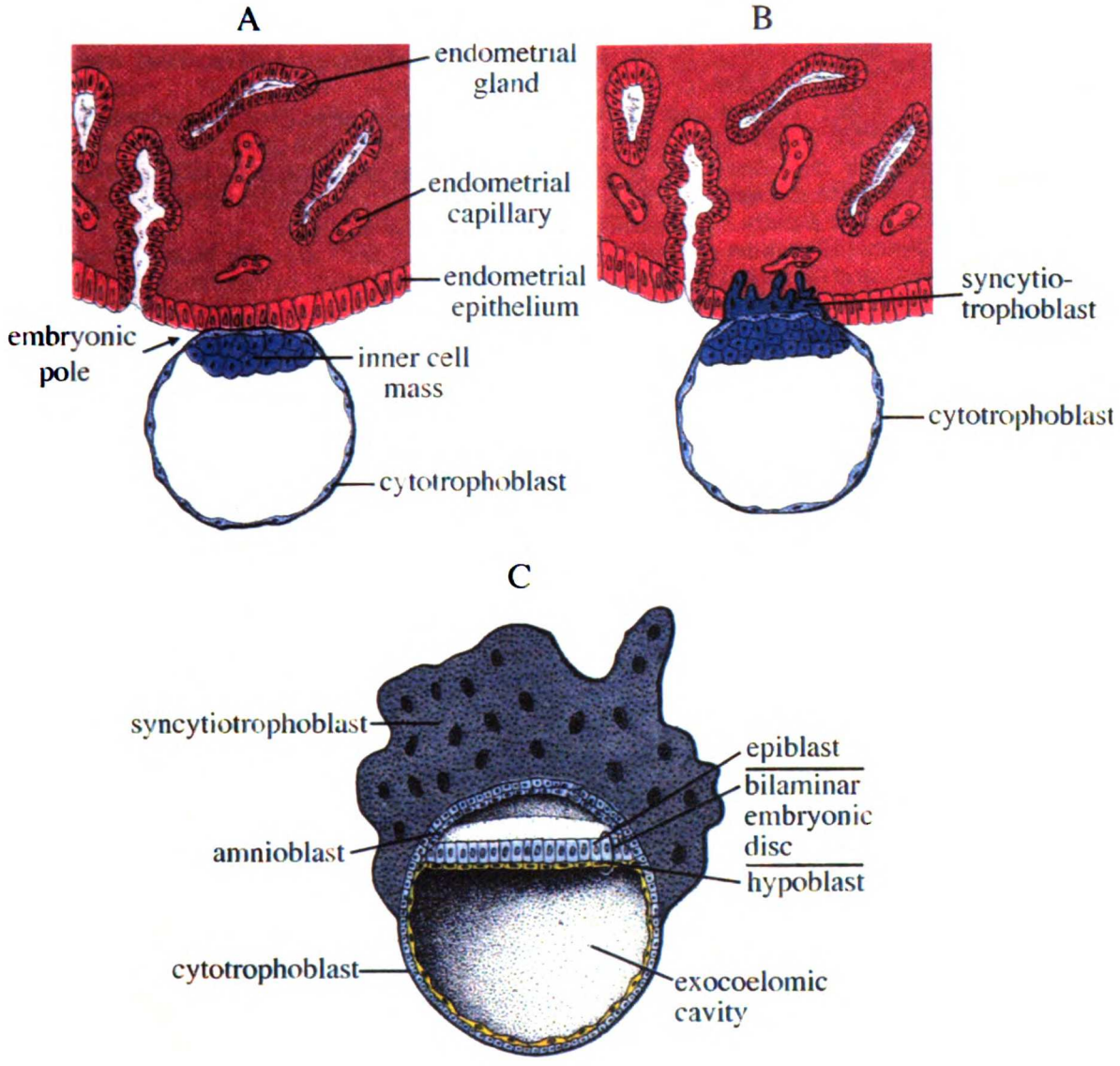

A
endometrial gland
endometrial capillary
endometrial epithelium
embryonic pole
inner cell mass
cytotrophoblast
B
syncytio-trophoblast
cytotrophoblast
C
syncytiotrophoblast
epiblast
bilaminar embryonic disc
amnioblast
hypoblast
cytotrophoblast
exocoelomic cavity

Figure 1-1

Figure 1-2: Early stages of chorionic villous development. A, Day 10: lacunae have formed within the syncytiotrophoblast, separated by syncytial trabeculi. B, Day 12-15: cytotrophoblasts at the chorionic plate proliferate and extend through the trabeculi, toward the endometrium. C, Day 13-16: cytotrophoblasts break through the syncytium at the distal tips of the trabeculi and migrate through the endometrium to surround the conceptus, forming a cytotrophoblastic shell. Cytotrophoblasts fill the early trabeculi to form primary chorionic villi. Later, mesenchyme appears in the core of some secondary villi. (Modified from Benirshke and Kaufmann, 1995, with permission).

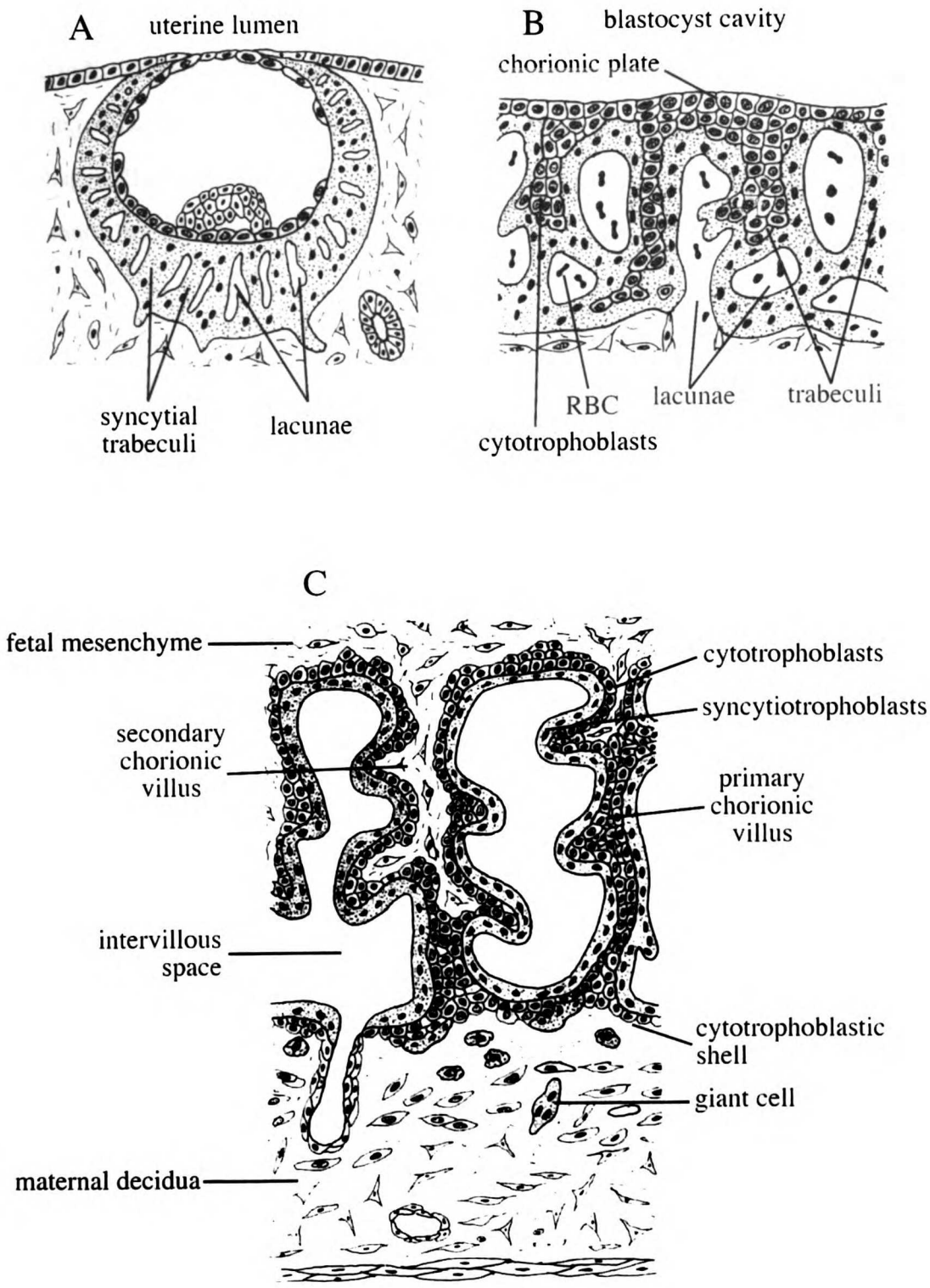

A
uterine lumen
syncytial trabeculi
lacunae
B
blastocyst cavity
chorionic plate
RBC
lacunae
trabeculi
cytotrophoblasts
C
fetal mesenchyme
secondary chorionic villus
intervillous space
maternal decidua
cytotrophoblasts
syncytiotrophoblasts
primary chorionic villus
cytotrophoblastic shell
giant cell

Figure 1-2

Figure 1-3: Drawings illustrating tertiary chorionic villi. A, Sagittal section of an embryo at about 21 days gestation. Fetal capillaries are visible within the stromal core of tertiary corionic villi. These capillaries are connected with the embyonic heart via vessels in the connecting stalk. B, Section of a tertiary chorionic villus. Fetal capillaries, as well as macrophages (Hofbauer cells), are seen within the stromal core. (Modified from Moore and Persaud, 1993, with permission).

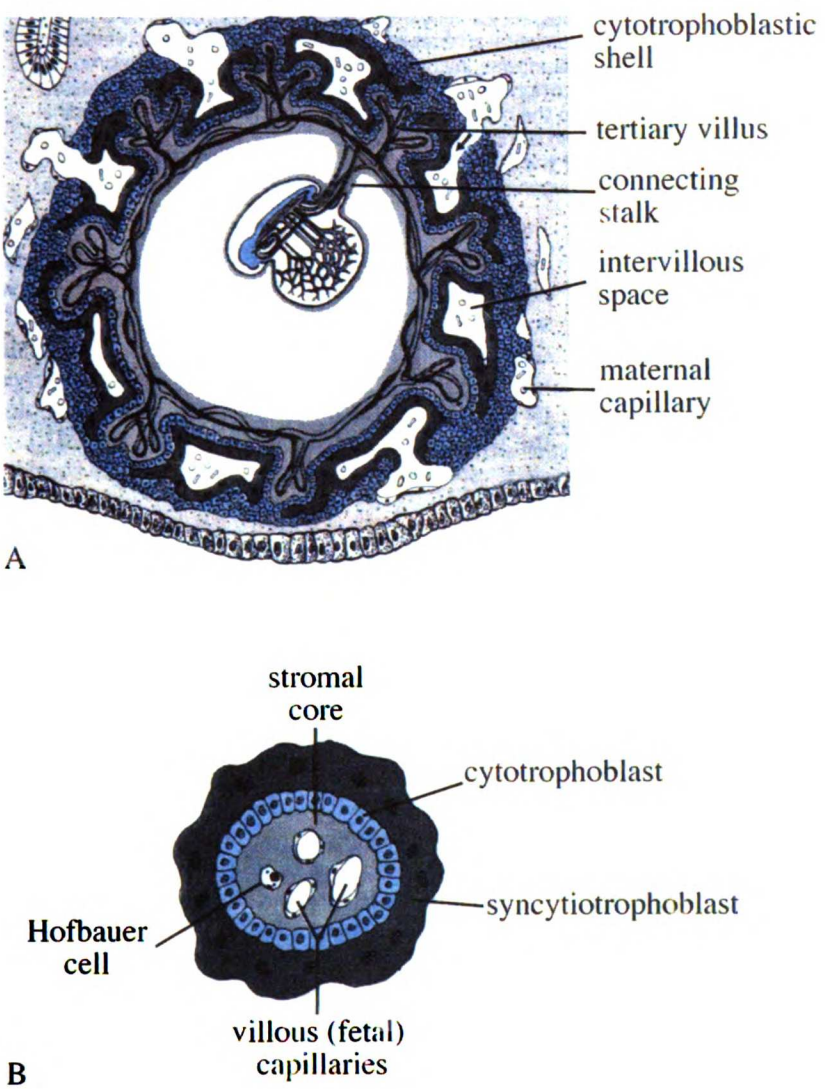

cytotrophoblastic shell
tertiary villus
connecting stalk
intervillous space
maternal capillary
A
stromal core
cytotrophoblast
syncytiotrophoblast
Hofbauer cell
villous (fetal) capillaries
B

Figure 1-3

Figure 1-4: Histology of mature chorionic villi. A, Multinucleated syncytiotrophoblasts overlie a monolayer of cytotrophoblasts, which surround the villous stromal core in both anchoring (AV) and floating (FV) villi. During interstitial invasion, cytotrophoblasts at the distal tips of anchoring villi form invasive columns of cells that migrate through the uterine wall. B, During endovascular invasion cytotrophoblasts invade the tunica media of maternal spiral arteries.

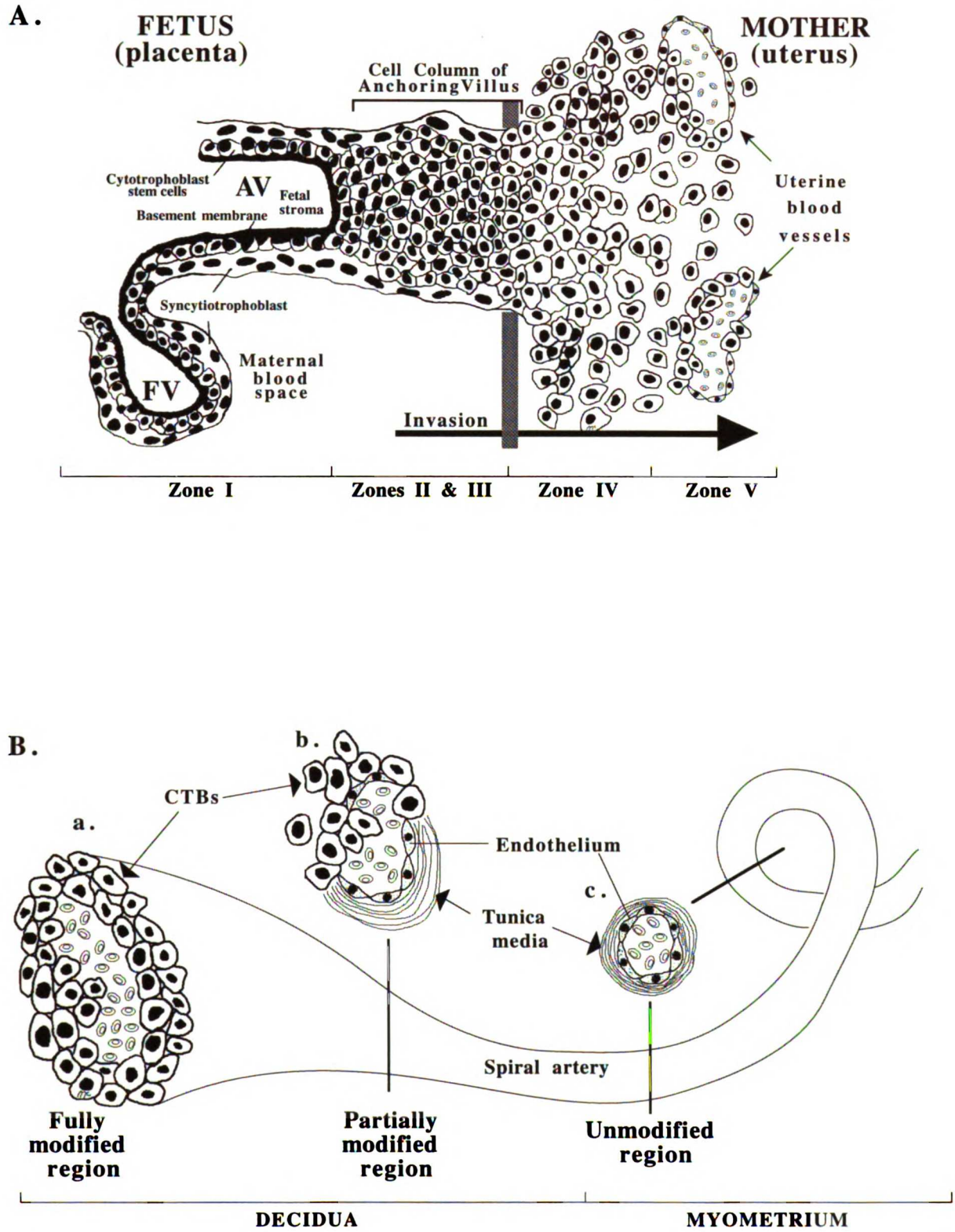

A.
FETUS
(placenta)
MOTHER
(uterus)
Cell Column of
AnchoringVillus
Cytotrophoblast
stem cells
AV
Fetal
stroma
Basement membrane
Uterine
blood
vessels
Syncytiotrophoblast
Maternal
blood
space
FV
Invasion
Zone I
Zones II & III
Zone IV
Zone V
B.
b.
CTBs
a.
Endothelium
c.
Tunica
media
Spiral artery
Fully
modified
region
Partially
modified
region
Unmodified
region
DECIDUA
MYOMETRIUM

Figure 1-4

Figure 1-5: Development of the placenta and fetal membranes. A, Section of the uterus showing a fetus at 5 weeks gestation within the posterior wall, and the association of fetal and maternal tissues. B, In the 10 week fetus, the villi at the decidua capsularis have regressed, forming the chorion laeve. C, The definitive placenta is composed of remaining chorionic villi and the decidua basalis. As the fetus grows , the amniotic sac fuses with the chorion laeve to form the amniochorionic membrane. F, By 22 weeks gestation, the amniochorionic membrane adheres to the decidua parietalis, thus obliterating the uterine lumen. (Modified from Moore and Persaud, 1993, with permission).

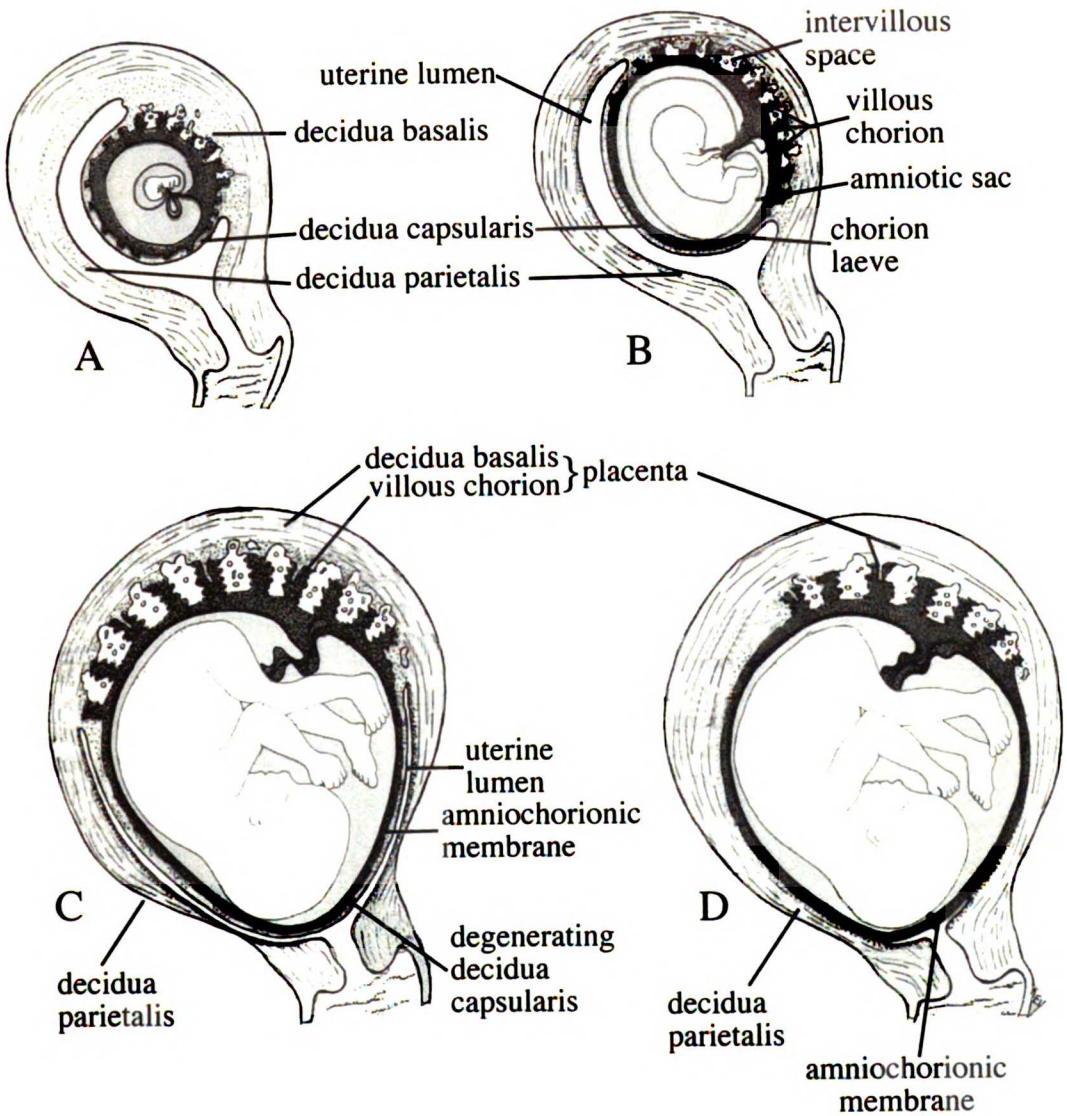

uterine lumen
decidua basalis
decidua capsularis
decidua parietalis
A
intervillous space
villous chorion
amniotic sac
chorion laeve
B
decidua basalis
villous chorion
placenta
uterine lumen
amniochorionic membrane
degenerating decidua capsularis
C
decidua parietalis
D
decidua parietalis
amniochorionic membrane

Figure 1-5

Figure 1-6: Anatomy of the mature human placenta. Chorionic villi grow from the chorionic plate at the chorion frondosum, toward the basal plate at the decidua basalis. Villi extend into the intervillous space, which is filled with blood from uterine spiral arteries. Floating villi are suspended within maternal blood, while anchoring villi attach to the basal plate and invade the decidua. (Modified from Benirschke and Kaufmann, 1995, with permission).

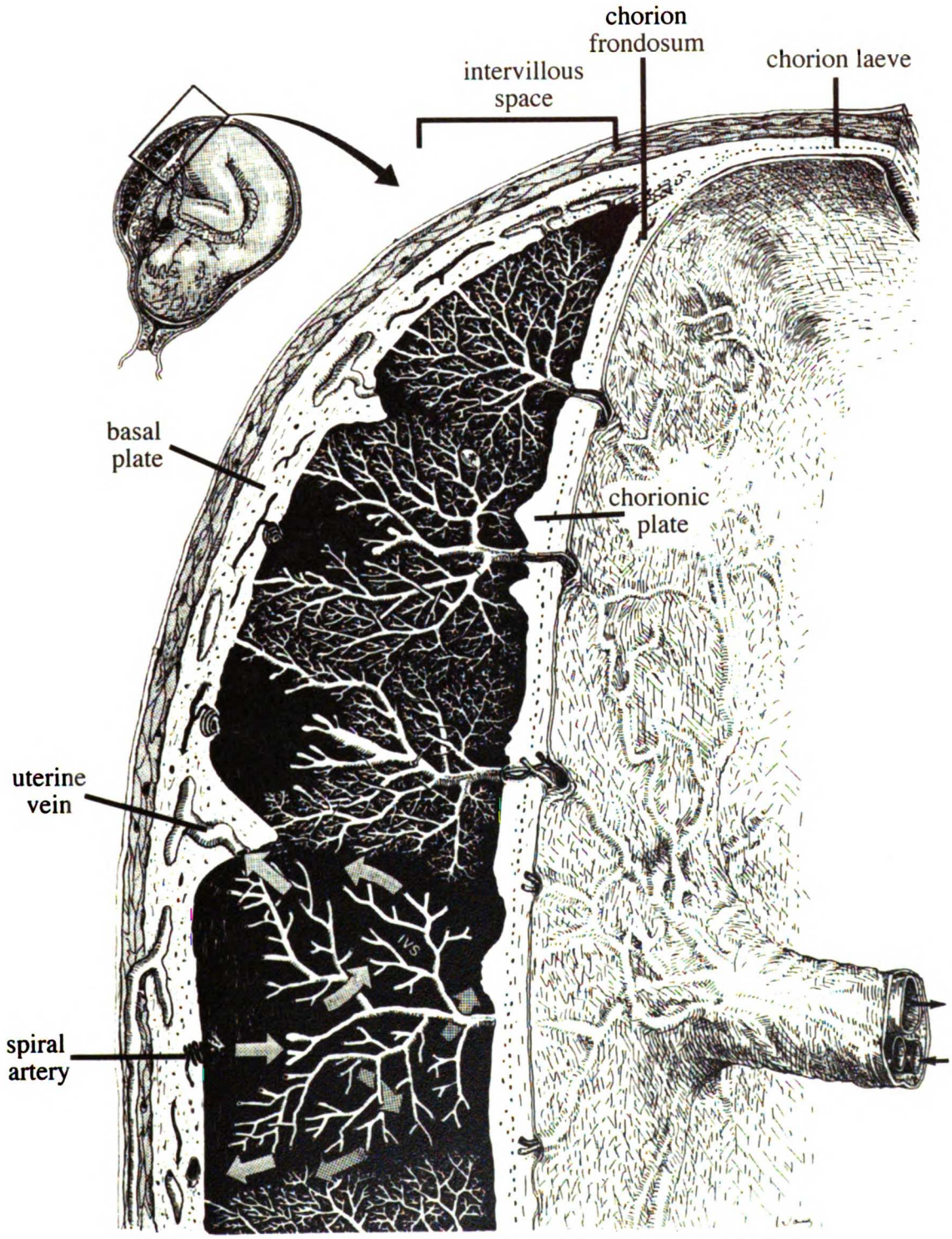

chorion
frondosum
chorion laeve
intervillous
space
basal
plate
chorionic
plate
uterine
vein
spiral
artery
IVS

Figure 1-6

Figure 1-7: Extensive branching of chorionic villous trees. Truncus chorii grow from the chorionic plate then branch into rami chorii of the first through fourth order (I, II, III, IV). Rami chorii continue to branch into 2 to 30 generations of slender ramuli chorii, a subset of which are anchored to the basal plate. Other villi float within the intervillous space and perform physiologic exchange between fetal and maternal circulations. (Modified from Benirschke and Kaufmann, 1995, with permission).

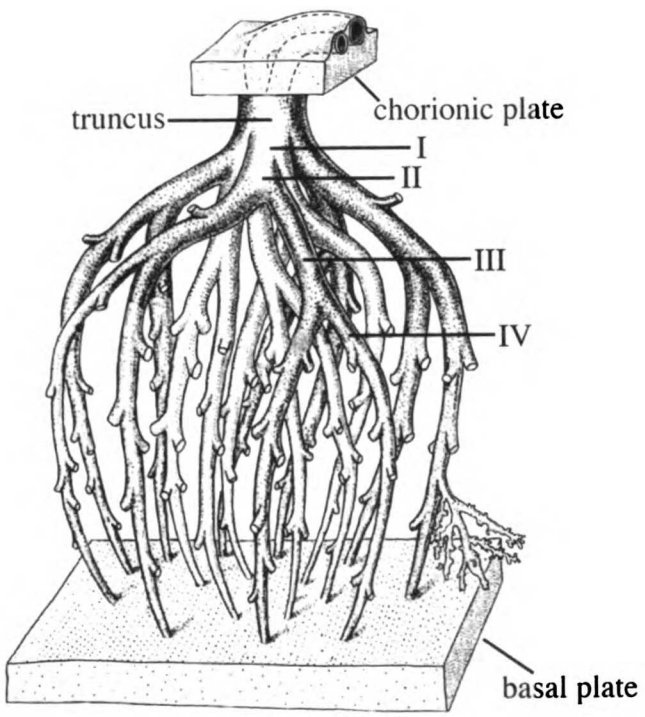
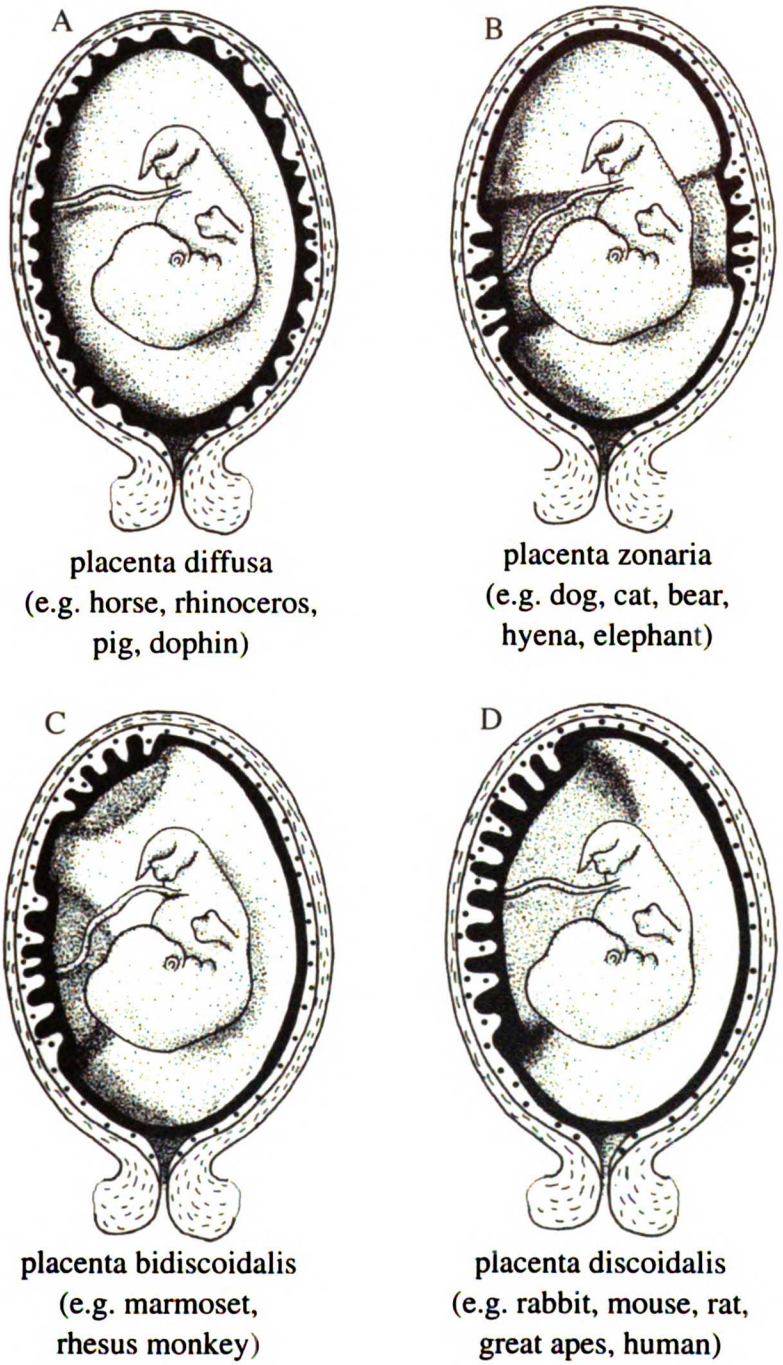


Figure 1-7

Figure 1-8: Placental shapes. A, In the placenta diffusa, fetal and maternal tissues are interdigitated over the entire uterine wall. B, The placenta zonaria forms a ring around the developing fetus. C, The placenta bidiscoidalis is composed of two distinct areas of fetal-maternal contact. D, The placenta discoidalis forms in one concentrated area. (Modified from Benirschke and Kaufmann, 1995, with permission).



placenta diffusa
(e.g. horse, rhinoceros,
pig, dophin)

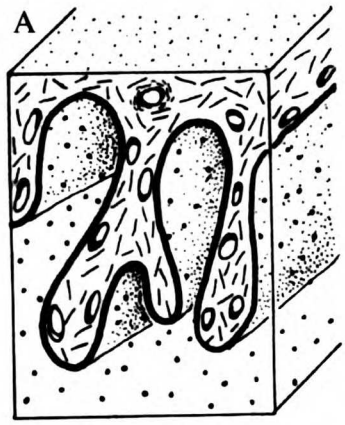
placenta zonaria
(e.g. dog, cat, bear,
hyena, elephant)

placenta bidiscoidalis
(e.g. marmoset,
rhesus monkey)

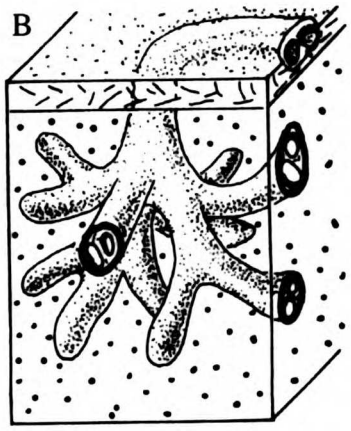
placenta discoidalis
(e.g. rabbit, mouse, rat,
great apes, human)

Figure 1-8

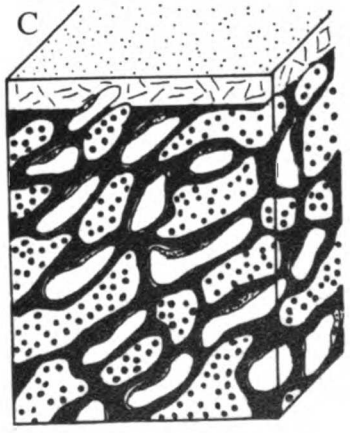
Figure 1-9: Types of interdigitation of fetal and maternal tissues. A, The folded placenta provides a small increase in surface area relative to planar apposition. B, In the villous placenta, branches of fetal chorionic villi float within a pool of maternal blood. C, The labyrinthine placenta is the most efficient form of exchange, with multiple fetal and maternal blood channels in close apposition within trophoblast tissue. (Modified from Benirschke and Kaufmann, 1995, with permission).



folded placenta
(e.g. pig, galago)



villous placenta
(e.g. rhesus monkey,
great apes, human)



labyrinthine placenta
(e.g. rabbit, mouse, rat)

Figure 1-9

Figure 1-10: Fetal-maternal blood barriers. In the endotheliochorial placenta, fetal and maternal blood are separated by fetal endothelial, trophoblast, and maternal endothelial cell layers. Either fetal cyto- or syncytio- trophoblast cells are in direct contact with maternal blood in hemochorial placentas. (Modified from Benirschke and Kaufmann, 1995, with permission).

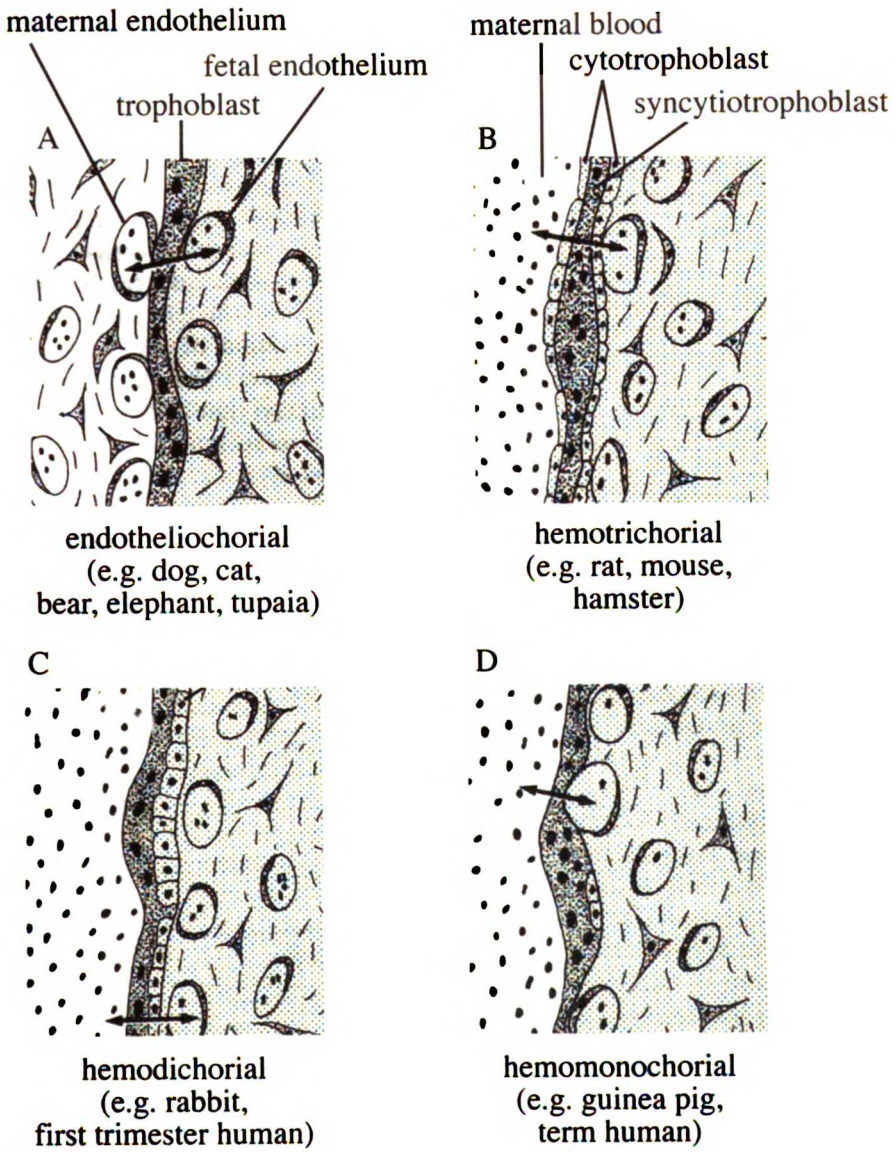

maternal endothelium
fetal endothelium
trophoblast
A
endotheliochorial
(e.g. dog, cat,
bear, elephant, tupaia)
maternal blood
cytotrophoblast
syncytiotrophoblast
B
hemotrichorial
(e.g. rat, mouse,
hamster)
C
hemodichorial
(e.g. rabbit,
first trimester human)
D
hemomonochorial
(e.g. guinea pig,
term human)

Figure 1-10

CHAPTER TWO

The Immunology of Pregnancy

General Introduction and Historical Perspective

In the human hemochorial placenta, the trophoblast is the only fetal cell type directly exposed to maternal cells: syncytiotrophoblasts are constantly being bathed by maternal intervillous blood, while invasive cytotrophoblasts mix with a variety of cell types in the decidua, up to 75% of which are immune-competent bone marrow-derived cells (Starkey et al., 1988). Since trophoblast cells are fetal in origin, they express a subset of paternal antigens that are genetically foreign to the mother. Although these semi-allogeneic trophoblasts are exposed to maternal leukocytes in intervillous blood and the decidualized endometrium, they are not rejected by the maternal immune system. Because successful pregnancy is essential to the propagation of mammalian species, it is unlikely that a single hypothesis will explain fetal evasion of maternal immune rejection. Instead, it is reasonable to expect that multiple redundant mechanisms have evolved to protect the conceptus and ensure that reproduction succeeds. Our knowledge of this field has grown significantly since the immune paradox of viviparity was first described three quarters of a century ago.

As early as 1924, geneticist C.C. Little recognized the apparent tolerance of the allogeneic embryo in mammalian pregnancy (Little, 1924). At that time, new studies in tissue transplantation raised the issue of genetic distinction among individuals, bringing to light the immune paradox of pregnancy. In a classic lecture from 1953, Nobel laureate Peter Medawar first described the problem of viviparity and formally introduced the field of pregnancy immunology (Medawar, 1954). In his report, Medawar asks, "How does the pregnant mother contrive to nourish within itself, for many weeks or months, a foetus [sic] that is an antigenically foreign body?". Among his answers were the ideas that the fetus and mother are separated anatomically, the fetus is antigenically immature, and the mother is immunologically indolent or inert. To these hypotheses, a former student of Medawar's later added that the uterus is an immunologically privileged site (Billingham, 1964).

Together, these theories have provided the fundamental basis for further work in the field. To begin to explain the unique and complex immunological state of pregnancy, several basic concepts in immunology must be understood.

Types of immune mechanisms

The modern definition of immunology is the study of the cellular and molecular events that occur after an organism encounters foreign substances, including microbes and transplanted tissues (reviewed in Abbas et al., 1994). Healthy individuals utilize two mechanisms to protect themselves against foreign substances, including innate (also called natural or native) and acquired, or specific, immunity (Figure 2-1). Components of innate immune reactions are present prior to exposure to foreign substances and, therefore, do not discriminate among them. This generalized immune response utilizes physical barriers (i.e. skin), phagocytic cells, natural killer (NK) cells, and serum complement proteins to protect the organism from foreign substances. Because they are not specifically targeted against a single substance, these responses do not change in magnitude after repeated encounters with the same foreign antigen. That is, innate immunity is nonspecific and non-adaptive.

The specific immune response utilizes the cooperative interactions of numerous cells and molecules, including lymphocytes and antibodies, for host defense. In contrast to innate immunity, the specific immune system is characterized by memory of each foreign antigen exposure. Subsequent encounters with the same antigen are not only augmented, but are focused to the antigenic site of entry, making its elimination more efficient. Specific immune responses generally require the participation of the components of innate immunity (Fearon and Locksley, 1996). In fact, specific immunity tends to enhance innate immunity, as reflected in their phylogeny. Prior to vertebrate evolution, host defense was mediated largely by innate reactions. Later, vertebrates developed specific immunity to

enhance these existing defenses. As will be discussed in the following sections, evidence suggests that innate and specific immune mechanisms are intact in the pregnant uterus, thus the placenta has developed ways to shield itself from both.

Specific immunity is usually stimulated by exposure to a foreign antigen in a process called immunization. This generates the induction of active immunity to protect the immunized host. Specific immunity can also be passively conferred upon another individual without direct exposure to antigen. Protection by passive immunity is acquired by the adoptive transfer of plasma or serum containing antibodies, or preformed immune cells.

Two types of specific immune responses, humoral and cell-mediated (or cellular) immunity, are characterized on the basis of the components that mediate the response. Humoral immunity involves antibodies, or immunoglobulins (Igs), molecules responsible for the specific recognition of different antigens, and can be transferred to naive individuals through plasma or serum. For instance, maternal antibodies routinely cross the placenta to confer passive immunity upon the developing fetus (Kristoffersen, 1996). Humoral immunity is the principal host defense against extracellular microbes because antibodies bind these and assist in their destruction. Cell-mediated immunity requires the participation of lymphocytes, specialized cells of the immune system that coordinate an active immune response. Therefore, this type of immunity can be adoptively transferred with cells, but not with serum. Intracellular microbes, such as viruses and some bacteria, are inaccessible to circulating antibodies and are eliminated by cell-mediated mechanisms. These include the induction of intracellular destruction of the microbes or lysis of the infected cells. Abnormal cells, such as tumor cells or foreign cells from tissue transplants, are also destroyed by these mechanisms. Antigenically foreign trophoblast cells are somehow protected from attack by both types of maternal immune responses.

The cellular basis of specific immunity

Humoral and cellular immunity are mediated by distinct types of lymphocytes that appear morphologically similar, but are quite different in their functions. One type consists of B lymphocytes, whose early stages of maturation occur in the bone marrow. B cells are specifically involved in humoral immune responses, as they are the only cell type capable of producing antibodies. The second type of lymphocyte is the T lymphocyte, which arise in the bone marrow but then migrate to and mature in the thymus. Different classes of T cells are involved in mediating both humoral and cell-mediated responses. Functionally distinct populations of lymphocytes express different membrane proteins. One group of cell surface proteins, known as cluster of differentiation (CD) markers, can be used to identify different classes of cells (Chan et al., 1988). The many different classes of leukocytes populating the pregnant uterus will be described in the sections that follow.

Both humoral and cell-mediated immune responses begin with the recognition of a foreign substance by a lymphocyte. Individual clones of lymphocytes express membrane receptors that can distinguish very subtle differences between distinct antigens. Those portions of the antigens that are specifically recognized are known as epitopes, or determinants. An individual's entire repertoire of epitope-specific lymphocytes develops during embryogenesis, without the prior exposure to the various antigens (reviewed in Sleckman et al., 1996). This diversity is the result of variability in the structure of the epitope binding site of lymphocyte receptors, resulting in a population of lymphocytes with an extremely diverse array of antigen receptors.

One of the most remarkable properties of the immune system is its ability to discriminate between self and foreign antigens. Tolerance to self antigens is acquired during lymphocytic development, at which time their encounter with antigens leads to their death

or inactivation (reviewed in Kruisbeek and Amsen, 1996; Melchers et al., 1995). Potentially self-recognizing lymphocytes come into contact with self antigens at a stage of functional immaturity, and are prevented from developing further. Because female fetuses could not be exposed to the antigens from placentas of future pregnancies, one would expect trophoblast-reactive lymphocytes to develop normally. Errors in either the initiation or the maintenance of self-tolerance can result in autoimmune diseases, where lymphocytes generate responses against self antigens (Goodnow, 1996).

Critical to the understanding of lymphocyte development is the clonal selection hypothesis, first proposed by Niels Jerne in 1955 then modified by Macfarlane Burnet in 1957 (reviewed in Talmage, 1986). This hypothesis states the following: 1) Every individual contains numerous clonally derived lymphocytes, each having arisen from a single precursor and capable of recognizing and responding to a distinct epitope. It is estimated that the mammalian immune system is capable of recognizing on the order of 10^9 distinct antigenic determinants. 2) Antigenic activation of a single, pre-existing clone induces lymphocyte proliferation and differentiation.. The expanded pool will subsequently respond much more rapidly to a second contact with the same antigen.

In humoral immunity, antigenic recognition by a B lymphocyte results in its proliferation, then differentiation into a clone of plasma cells. Each clone of plasma cells produces antibodies with the identical specificity as those on the surface of the founding B cell. However, plasma cells produce a secreted form of the antibodies, which bind to antigens to trigger effector functions of the humoral immune system. When bound to certain toxic or infectious proteins, secreted antibodies can sterically hinder their harmful effects, thus essentially neutralizing the antigen.

Antigen binding by antibody molecules can also activate the complement pathway, a major effector mechanism for humoral immunity (Tomlinson, 1993). In the classical pathway, antigen-antibody complexes initiate a cascade of proteolytic degradations of serum zymogens. In the parallel alternative pathway, some complement components can be activated by binding directly to the surfaces of infectious organisms. Complement activation thus contributes to innate immunity, in addition to specific immune mechanisms. Both classical and alternative pathways converge and lead to the formation of the membrane attack complex (MAC). This lipid soluble pore structure mediates osmotic lysis of cells.

T cell activation induces their differentiation into several classes that are involved in the regulation of both humoral and cellular immune responses. One type is known as a T helper (Th) cell, and is characterized by their expression of the CD4 marker (Mosmann and Sad, 1996). $CD4^+$ Th are further subdivided into two populations, distinguished on the basis of different profiles of cytokine production. Cytokines are soluble protein hormones that direct and modulate different immune responses. Th2 cells are important for mediating humoral immune reactions by secreting cytokines, such as interleukin (IL) -10 and -4, that are required for the differentiation of B cells into antibody-producing plasma cells.

$CD4^+$ Th1 cells secrete proinflammatory cytokines to provide help for three types of cell-mediated immune reactions. In delayed type hypersensitivity (DTH), the secretion of tumor necrosis factor (TNF) helps recruit blood monocytes to sites of antigen challenge. Monocytes are members of the mononuclear phagocyte system and are another major cell population of the immune system, in addition to lymphocytes. Interferon (IFN) -γ secretion by Th1 cells stimulates monocytes to differentiate into macrophages, which eliminate the antigen. Activated macrophages phagocytose and kill microorganisms as the primary defense mechanisms against intracellular bacteria. As a link between humoral and specific immune mechanisms, the coating of antigenic particles with antibody molecules, in

a process called opsonization, enhances phagocytosis by phagocytes by strengthening their attachment to the target antigen.

In the second type of cell-mediated immune response, $CD4^+$ Th1 cytokines IL-2 and IFN-γ stimulate the differentiation of $CD56^+$ NK cells. Activated macrophages also secrete IL-12 to further enhance NK stimulation (Trinchieri, 1995). These lymphocytes, distinct from both B and T cells, are large cells containing numerous cytoplasmic granules. Activated NK cells differentiate into lymphokine-activated killer (LAK) cells, which lyse target cells in an antigen-non-specific manner. Killing by LAK cells involves exocytosis of their numerous cytoplasmic granules to release the pore-forming protein, perforin (Kagi et al., 1996). Exocytosis of perforin is focused on the target cell, and causes ion-permeable channels to form in its plasma membrane, leading to osmotic swelling and lysis . The result is an effective way to destroy cells infected with intracellular pathogens, as well as abnormal tumor cells and foreign cells from a genetically dissimilar individual. Because of their lack of antigen specificity, NK cells represent a part of the more primitive innate immune system, and demonstrate how specific immunity can enhance innate immune function (Trinchieri, 1995). Although NK cells do not have specificity for particular antigenic determinants, their pattern of lysis is not random. The mechanisms of NK target selection are the subject of ongoing investigation, and will be discussed in the sections that follow with regards to NK recognition of trophoblast cells.

$CD4^+$ Th1 cells also participate in a third type of cell-mediated immune reaction. In this response, Th1 cytokines provide a signal for the differentiation of a second class of T cell, distinct from Th cells, known as cytotoxic T lymphocyte (CTL; Berke, 1994). The vast majority of CTLs express the CD8 marker of differentiation, although rare CTLs are $CD4^+$. $CD8^+$ CTLs directly perform effector cell functions for host defense against virally infected cells, or foreign, or otherwise abnormal, cells in acute allograft and tumor

rejection. To eliminate the target, CTLs kill cells by mechanisms similar to those used by NK cells, namely exocytosis of granules containing the cytotoxic protein, perforin (Berke, 1994). Some have suggested that the interaction of Fas ligand, on the surface of the activated CTL, with the apoptosis-inducing Fas molecule, on the target cell, may also be important for CTL killing. However, evidence for a role of Fas-mediated cytotoxicity in host defense in vivo is lacking (Kagi et al., 1996). Unlike NK killing, CTL lysis is antigen-specific such that each clone of cells is directly targeted to one antigenic epitope.

Some believe that a third class of T lymphocyte, distinct from Th cells and CTLs, exists. Like other cellular members of the specific immune response, these suppressor T cells function in an antigen-specific manner (Dorf and Benacerraf, 1984). These cells are unique in that they are thought to directly inhibit the activation of T and/or B lymphocytes. This function may be important in preventing immune responses to self antigens that are not accessible to immature lymphocytes, and therefore cannot induce tolerance. Some believe that suppressor T cells play an important role in the pregnant uterus. Unfortunately, progress in the characterization of suppressor T cells and their secreted factors has been slow, therefore many doubt their existence. The more recent definition of complex cascades of interacting T cell subsets and various soluble mediators can explain most of the effects once attributed to T suppressor cells. Thus, work in this field remains very controversial (O'Hara, 1995).

Regulation of immune responses

One of the cardinal features of the immune system is the self-regulation of normal responses. Immune responses are generally self-limiting, waning with time after antigenic stimulation. Stimulated lymphocytes perform their functions for only brief periods, after which they either become memory cells, or differentiate into short-lived effector cells. In

addition, ongoing immune responses stimulate a number of feedback mechanisms that are autoregulatory for the response itself. Because the presence of antigen activates an immune reaction, its eventual elimination subsequently removes the stimulus for further lymphocyte activation. Finally, the products of lymphocyte activation, namely antibodies and cytokines, also have short half-lives and are secreted only for brief periods immediately following antigenic stimulation.

Cytokines are secreted by T lymphocytes and mononuclear phagocytes to regulate immune responses (reviewed in Howard et al., 1993). Cytokines include a diverse array of protein mediators originally identified as products of these immune cells. Cytokines are involved in cell growth, inflammation, immunity, and differentiation by exerting both stimulatory and inhibitory effects on different cell types. One unifying feature of all cytokines is their extensive pleiotropy and their redundancy: each cytokine has multiple functions, and any one function is generally mediated by more than one cytokine. This redundancy makes cytokine networks extremely difficult to understand, as well as making data difficult to evaluate. Redundancy within the cytokine family is well highlighted with the recent development of mutant mice which have disrupted individual cytokine genes, or "knockout" mice. Although such mice sometimes exhibit defects that can be predicted from some known functions of the disrupted cytokine, it appears that alternative cytokines often compensate for many additional functions.

Unlike hormones, which are released into the bloodstream and circulated throughout the body, cytokines are usually involved in local effects. These effects can be both paracrine, having local effects on a different cell type, and autocrine, acting on the cell by which it was synthesized. Cytokines mediate their effects by binding to high affinity specific receptors that usually belong to a family of structurally homologous receptor types. Both cytokines and their receptors are expressed transiently following immune stimulation, thus

regulating their activity temporally. Cytokine synthesis can also be influenced by other cytokines, resulting in regulatory loops that can either amplify or inhibit a response.

The control of humoral and cell-mediated immune responses by T helper cells provides an example of the complexity of cytokine regulatory networks. The two $CD4^+$ Th cells subsets, Th1 and Th2, are characterized by their cytokine secretion profile and immune function (Mosmann et al., 1986; Mosmann and Coffman, 1989) Th1 cells produce the proinflammatory cytokines IL-2 and IFN-γ, and act to induce cell-mediated immunity (Cher and Mosmann, 1987). Th2 cells secrete IL-4 and IL-10, cytokines that stimulate humoral immune reactions (Mosmann and Moore, 1991). While this phenomenon was originally described in the mouse, evidence suggests that human $CD4^+$ T cells can be classified into similar subsets, including a third, Th0, which is characterized by the secretion of both Th-1 and -2 cytokines (Romagnani, 1991). These Th-mediated immune responses are mutually exclusive due to the reciprocal inhibition of each subset (reviewed in Howard et al., 1993). In addition to stimulating cellular immunity, the Th1 product, IFN-γ, is directly inhibitory for antigen-stimulated growth of Th2 cells. In contrast, Th2 cells secrete IL-10 and IL-4 to stimulate humoral immunity and suppress Th1 cytokine production, thus inhibiting Th1-mediated cellular immune reactions.

One of the best studied examples of the Th1/Th2 dichotomy is the murine response to infection with the intracellular parasite, *Leishmania major* (reviewed in Locksley and Scott, 1991). In BALB/c strains, infection with *L. major* causes a localized lesion, but the disease quickly disseminates to visceral organs and the mouse succumbs to a systemic infection. In contrast, mice of other strains completely resolve the infection within a few weeks. In both cases, $CD4^+$ Th cells are stimulated, although the nature of the response is quite different between the two strains. The healing response is associated with a strong DTH reaction, mediated by the secretion of high levels of the Th1 cytokine, IFN-γ. In

contrast, responding BALB/c T lymphocytes differentiate into Th2 cells, which secrete high levels of IL-4. IL-4 inhibits the secretion of IFN-γ, consequently preventing the stimulation of Th1-mediated effector mechanisms necessary to clear intracellular infections. A similar correlation between cytokine profiles and disease outcome has also been observed clinically in a variety of human diseases (reviewed in Howard et al., 1993). By secreting different cytokine profiles, Th cells thus help activated lymphocytes generate either a humoral or a cell-mediated immune response.

Mechanisms of lymphocyte activation by foreign antigen

The antigenic requirements for the initial activation of B and T lymphocytes are distinct. B cells produce antibodies in a membrane-bound form, and these Igs function as the B cell antigen receptor (Desiderio, 1992). B lymphocytes, and the antibodies they secrete after activation, are capable of directly recognizing a variety of antigenic types, including proteins, carbohydrates, nucleic acids, or lipids under the appropriate conditions. In addition, the B cell receptor and antibodies can interact with either intact, denatured, or even degraded molecules, and the interaction is conformation-dependent.

In contrast, T cells respond exclusively to protein antigens, and do not recognize free antigen. T cells require the denaturation and degradation of antigens, and their subsequent presentation on the cell surface (reviewed in Rammensee, 1996). T cells are specifically stimulated by a non-covalently linked complex of short polypeptide fragments of the degraded antigen and special cell-surface molecules. These cell-surface molecules are encoded by closely linked genes in the 3500 kb major histocompatibility complex (MHC) on the short arm of chromosome 6 (Klein et al., 1993). Several loci encode two classes of MHC molecules, each presenting a different class of antigens.

MHC class I molecules include the highly polymorphic transplantation antigens, human leukocyte antigens (HLAs) -A, -B, and -C. At least 24 different alleles of HLA-A and 50 alleles of HLA-B have been identified in the human population. The actual number of functionally distinct alleles may even be substantially higher. In addition, each person inherits a copy of chromosome 6 from both their mother and father, and can thus express up to 6 different class I HLA-A, -B, and -C proteins. The HLA complex is the most polymorphic gene system known and can present a major problem in transplantation. These classical class I molecules differ from the nonclassical MHC class I-like molecules, HLA-E, -F, and -G, in that the latter group displays limited polymorphism.

All class I molecules contain two separate polypeptide chains: an α chain, encoded by the MHC locus, and an invariant ß chain, called $ß_2$ microglobulin (York and Rock, 1996; Figure 2-2). Only the α chain of HLA-A, -B, and -C displays a high degree of polymorphism. Class I molecules are expressed on virtually every nucleated cell, and present peptides of 9-11 amino acids derived from antigens synthesized within the cell. Antigens associated with all class I HLA molecules are recognized by antigen-specific $CD8^+$ CTLs whose primary function is to eliminate intracellular pathogens. This population of lymphocytes is also involved in the elimination of abnormal cells, including virally-infected and tumor cells, and non-self cells in tissue transplants. CTLs recognize foreign peptide bound to self class I MHC or, the case of a transplant, foreign class I molecules on the target cell surface and specifically eliminate that cell.

The three class II molecules, HLA-DP, -DQ, and -DR, are also composed of two polypeptide chains, the α and ß chains (Cresswell, 1994). Unlike class I HLA molecules, both chains of the class II molecules are encoded by polymorphic sequences in the MHC locus. Class II MHC molecules are expressed on the surfaces of antigen presenting cells (APC). This population comprises a more limited repertoire of cells, including some

monocytes and epithelial cells, many tissue macrophages, and all B cells. These cells endocytose extracellular proteins, degrade them in special endosomes, then present 10-30 amino acid-sized fragments complexed to class II MHC molecules on the cell surface. Extracellular peptides associated with class II MHC activate $CD4^+$ Th cells to begin the generation of an immune response.

The T cell receptors that recognize peptide-MHC complexes are disulfide-linked heterodimeric proteins that share homology with Ig molecules (Figure 2-3). The majority of T cells express receptors composed of α and ß chain subunits (αß T cell receptor; TCR), although a small subset of αß-negative T cells express $\gamma\partial$ TCRs (Moretta et al., 1991). Both $CD4^+$ and $CD8^+$ αß T cells have been described, while the majority of $\gamma\partial$ cells lack surface CD4 and CD8. Unlike αß TCR cells, $\gamma\partial$ cells are homogeneously composed of cytolytic precursors. Both αß and $\gamma\partial$ TCRs are noncovalently associated with a complex of up to five distinct, invariant membrane proteins, collectively called the TCR complex. These include the CD3 proteins, composed of γ, δ, and ε chains that most likely exist as monomers within the TCR complex. 90 percent of TCR complexes additionally contain a homodimer of ζ chains, while the remaining 10 percent contain a $\zeta\eta$ heterodimer. Together, this complex of integral membrane proteins may function as the signal-transducing element of the TCR.

In addition to TCR:CD3, T cell stimulation requires ligand binding by several accessory molecules (Figure 2-4). CD8 and CD4 are accessory molecules expressed on mutually exclusive subsets of T lymphocytes (CTL and Th, respectively), that bind monomorphic determinants of class I and class II MHC molecules, respectively (Leahy, 1995). CD28 on the T cell surface binds the costimulatory molecule B7 on APCs during T cell activation (June et al., 1990). Some T cell accessory molecules are integrin cell adhesion receptors that bind ligands on APCs to stabilize cell-cell contacts. An example of this type of T cell

receptor are members of the lymphocyte function-associated antigen (LFA), or integrin ß2, family (Westphal et al., 1993). Family members are formed by the noncovalent interactions between three distinct α chains (CD11a, -b, and -c) and the same ß chain (CD18). LFA-1, composed of CD11a and CD18, binds intercellular adhesion molecule (ICAM) -1 on the surface of activated cells. LFA-2 (CD2), on the surface of T lymphocytes and NK cells, forms adhesive interactions with LFA-3 (CD58).

Accessory molecules are important in influencing the functional interactions between T cells and the antigen presenting of target cells. However, T cell activation is the direct result of binding of the antigen-MHC complex to the TCR:CD3 complex. This interaction initiates a series of events, beginning with the transduction of an intracellular signal that leads to the transcriptional activation of a variety of genes (Cantrell, 1996). New cell surface molecules are then expressed on the activated T cell, and cytokines are secreted to direct the activity of additional immune cells. The stimulated cell also proliferates in response to foreign antigen, resulting in a clonal expansion of antigen-specific T cells. Different populations of T cells display unique effector functions after activation: $CD4^+$ Th cells secrete cytokines, while $CD8^+$ CTLs lyse target cells directly.

In contrast to αß T cells, very little is known about the biological function of γδ TCR-expressing lymphocytes (reviewed in Chien et al., 1996). Recent work has suggested that γδ cells may not recognize antigen the same way as do αß T cells. Studies with murine γδ cells shows that antigen recognition by this subtype does not require antigen processing, and that proteins can be recognized directly. In this regard, γδ T cells may be more like Igs. Evidence also exists for the participation of MHC molecules, particularly the non-classical class I molecules, in antigen recognition by γδ cells (reviewed in Kronenberg, 1994). γδ T cells can also be stimulated in a TCR-dependent manner with nonpeptide compounds derived from mycobacterium extracts. Although αß and γδ T cells recognize

antigens differently, they share many more characteristics, including cell surface protein expression (as described above), secretion of cytokines, and cytotoxic activity. This suggests that γδ T cells can mediate cellular immune functions with a greater flexibility than αß cells.

For the initiation of a humoral immune response, foreign antigens are first bound by the B cell receptor. After internalization of the antigen-receptor complex, the antigen is degraded and peptide fragments are presented on the surface of the B cell in association with class II MHC molecules. A subsequent MHC-restricted interaction between the B and $CD4^+$ T helper lymphocytes provides a costimulatory signal for B cell maturation (Parker, 1993). This interaction also leads to the secretion of Th2 cytokines by the T cell, which further augment antibody responses. A small subset of thymus-independent, or T cell-independent (TI), antigens can induce antibody responses without the participation of Th cells (Stein, 1992). One example is lipopolysaccharide (LPS), or endotoxin, a component of the cell walls of several gram-negative bacteria. (fig p191 abbas). Lymphocyte activation subsequently results in the mobilization of different immune cell subsets that function together to eliminate foreign microbes.

Immune recognition of foreign MHC

Tissue transplantation, dating back to ancient times, stimulates immune responses with the same mechanisms as do those used in host defense against foreign microbes. Exposure to allogeneic molecules through tissue transplantation is unlikely in the normal life of an organism. However, because the placenta is thought to express both maternal and paternal antigens, many have referred to it as an allograft. The maternal response to foreign trophoblast cells is not clearly defined, and is the subject of current investigation as will be reviewed in the following sections. In standard transplantation biology, the response to

allogeneic molecules is very strong, often involving every cell type of the immune system. Lymphocyte response to alloantigens expressed by transplanted tissues can be either cell-mediated or humoral, although cell-mediated immune reactions are more important for rejection.

Foreign MHC molecules are recognized by the host T cells to initiate graft rejection much like T cells recognize foreign peptide bound to self MHC (Sherman and Chattopadhyay, 1993). The TCR recognizes residues of both the presented peptide and the MHC molecule to which it is complexed. Because each foreign MHC differs from self MHC at multiple sites, recognition of different determinants by individual T cell clones results in widespread cross-reactivity with many T cell clones. In the same way, a variety of different peptides complexed to a single foreign MHC molecule, can elicit the activation of large numbers of T cell clones, each of which are specific to different peptide antigens. As a result, many more T cell clones can respond to a single foreign MHC molecule than to a specific foreign antigen. For this reason, alloreactive cells are very common and can quickly generate a very harmful immune response.

$CD8^+$ αß TCR CTLs recognize foreign class I MHC molecules and are activated to lyse the target cell (Wecker and Auchincloss, 1992). At least some $CD8^+$ γδ TCR cells are capable of recognizing polymorphic determinants of class I molecules and mounting cytolytic responses to alloantigens. As in other immune reactions, maximal CTL activity requires the cytokine products of $CD4^+$ T helper cells. Alloreactive $CD4^+$ Th cells are class II MHC-restricted and are stimulated only if there are differences in host and donor class II alleles. For this reason, allografts that contain class II MHC-expressing APCs stimulate strong rejection responses. Activated $CD4^+$ Th cells can subsequently recruit and activate macrophages, leading to graft injury by a DTH response as well. In addition to cell-

mediated immune attack, alloreactive B cells generate antibodies specific for the foreign cells, activating the complement system and injuring graft blood vessels.

The fetus and placenta do not exhibit the typical signs of graft rejection, suggesting that the maternal immune system does not mount similar responses during pregnancy. Medawar initially suggested that, because the fetus and mother are separated anatomically by intervening placental tissue, the fetus is protected from such a maternal immune attack. This idea does not explain the apparent survival of placental tissue, which is fetal in origin thus allogeneic as well. Recognizing that erythrocyte antigens are present several months before term, Medawar suggested that perhaps tissue antigens may develop later in life . The result is an antigenically immature fetus and, by extension, fetally-derived tissues such as the placenta. More recently, significant contributions to the field of reproductive immunology have increased our understanding of placental antigenicity.

The trophoblast as an allograft

The question of trophoblast antigenicity is central to the study of pregnancy immunology. Because trophoblast cells are the only fetal cell type in direct contact with maternal immune cells, whether they are recognized as an allograft is of great interest. It was first suggested in 1932 that villous trophoblasts do not express ABO blood group antigens and, therefore, function as a protective, nonantigenic barrier against immune rejection (Witebsky and Reich, 1932). After the discovery of human leukocyte antigens in the MHC locus, investigators began to look for MHC class I and II molecules on the surface of trophoblast cells using tissue typing allosera.

By 1976, the results of studies by both Faulk and Temple, and Goodfellow et al. concluded that human villous cytotrophoblast and syncytiotrophoblast do not express

classical MHC class I HLA antigens, and therefore may passively evade a maternal immune response (Faulk et al., 1977; Faulk and Temple, 1976; Goodfellow et al., 1976). Monoclonal antibody (mAb) technology was later developed and antibodies were raised against polymorphic determinants on class I and II molecules (Barnstable et al., 1978). These antibodies, coupled with new immunohistochemistry techniques, were used by many groups to confirm the absence of MHC class I and class II HLA antigens on the surface of trophoblast cells. The first evidence suggesting otherwise was the reaction of extravillous cytotrophoblasts with the W6/32 mAb, recognizing monomorphic determinants on class I molecules (Parham et al., 1979; Sunderland et al., 1981). The same cells were also shown to react with antibodies to ß-2–microglobulin, the invariant subunit of class I molecules (Ellis et al., 1986). The presence of class II HLA antigens on trophoblast cells has been consistently denied.

Cytotrophoblast reactivity with the W6/32 mAb was subsequently shown to be the result of their expression of unique, nonclassical class I HLA antigen, HLA-G (Ellis et al., 1990; Kovats et al., 1990). At approximately 39 kD, HLA-G is smaller than the 45-kD classical class I molecules due to the truncation of its cytoplasmic tail (Ellis et al., 1990; Geraghty et al., 1987). Whether HLA-G retains the signaling properties of classical class I molecules in spite of its shortened cytoplasmic tail is unknown. While HLA-G protein has not been detected in villous cyto- and syncytiotrophoblasts, some investigators have detected HLA-G mRNA in these cell populations (Hunt et al., 1990; Lata et al., 1992; McMaster et al., 1995). Amniotic membrane epithelial cells contain both class I mRNA and protein, but it is unknown whether this reactivity is due to the expression of a classical or a nonclassical class I molecule (Hunt et al., 1988). Invasive cytotrophoblasts in the decidua are the only trophoblastic cell type known to express both HLA-G mRNA and protein, as shown by immunohistochemistry with an HLA-G–specific mAb (McMaster et al., 1995; Yelavarthi et al., 1991). HLA-G mRNA was also detected in cytotrophoblasts within the chorion laeve

(Yelavarthi et al., 1991). These extravillous cytotrophoblasts also react with antibodies specific for nonpolymorphic, but not polymorphic, class I epitopes, suggesting that these cells express HLA-G (Hsi et al., 1984).

HLA-G was initially reported to be non-polymorphic, in stark contrast to the extensive array of allelic variation in classical class I molecules (Kovats et al., 1990). Because allogeneic antigens are not expressed on the surface of trophoblast cells, this observation negates the original definition of the fetus as an allograft. Thus, HLA-G was proposed to function as a universal "self" transplantation antigen, preventing maternal immune attack of the feto-placental unit (Redman, 1990). However, recent work has demonstrated HLA-G sequence variations resulting in amino acid subsitutions within a population of African Americans (van der Ven and Ober, 1994). To date, a total of approximately 30 different alleles have been described, indicating that HLA-G is a polymorphic gene (J. Phillips, personal communication). It is unclear whether HLA-G protein is expressed in the fetal thymus, which is important for the selection of lymphocyte clones capable of recognize HLA-G as a "self" MHC molecule (Shukla et al., 1990).

Studies to elucidate the function of cytotrophoblast HLA-G expression are ongoing, but this molecule may be important in mediating protective immune interactions between invasive cytotrophoblasts and maternal decidual leukocytes. Alternative splicing of HLA-G in choriocarcinoma cell lines yields secreted forms of the molecule (Ellis et al., 1990; Kovats et al., 1990). Soluble HLA-G produced by invasive cytotrophoblasts may bind maternal TCR in the absence of accessory molecules, and thus prevent their stimulation. Whether HLA-G presents antigen to the TCR is not known, but its potential to do so is supported by evidence that components of the peptide-MHC assembly apparatus are expressed in the placenta (Roby et al., 1994). HLA-G molecules have also recently been shown to be associated with a wide array of peptides derived from intracellular proteins

(Diehl et al, 1996). In addition, HLA-G has been shown to bind CD8, suggesting that CTLs can recognize HLA-G–bearing cells (Sanders et al., 1991).

Unlike lymphocytes, NK cells kill by an MHC-independent mechanism, but do not kill indiscriminately (Gumperz and Parham, 1995; Moretta et al., 1992). During development, NK cells adapt to self class I molecules on which they recognize epitopes shared by groups of class I alleles (Moretta et al., 1996). Most NK receptors recognizing MHC class I molecules on normal cells are inhibitory for cell killing, however an activating HLA-C specific receptor has been identified in some donors (Lanier and Phillips, 1996; Moretta et al., 1996). HLA-G may thus present only a limited array of peptides to inhibitory receptors on maternal NK cells. In fact, recent evidence suggests that expression of HLA-G by otherwise susceptible target cells prevents their lysis by activated NK cells (Pazmany et al., 1996).

HLA-G expression by invasive cytotrophoblasts may allow these cells to avoid classical allorejection by maternal leukocytes in the decidua. However, the expression of HLA-G by the relatively small number of invasive cytotrophoblast may not be sufficient to explain the apparent immune protection of the entire fetal allograft. While villous cytotrophoblasts and syncytiotrophoblasts do not express HLA-G, they are exposed to significant numbers of maternal leukocytes in the intervillous blood. In addition, this population of trophoblast cells is in large excess compared to invasive cytotrophoblasts in the placental bed. While the complete lack of class I expression does not induce CTL responses, it does result in NK-mediated lysis of the null cell (Ljunggren and Karre, 1990; Shimizu and DeMars, 1989). HLA-G–null trophoblast cells may thus be susceptible to immune attack by maternal NK cells. However, the placenta appears to be unharmed, suggesting that other protective mechanisms exist for the immunological protection of villous trophoblast cells.

The potential expression of as-yet-undefined class I HLA molecules by these cells is the subject of current investigation.

The placenta is also capable of protecting the fetus from the maternal humoral immune system. Antibodies against paternal antigens have been demonstrated in the sera of normal pregnant women (Aksel, 1992; McIntyre and Faulk, 1986). It is thought that the placenta acts as an immunoabsorbent barrier to prevent the passage of these potentially harmful antibodies into the fetal circulation (Wegmann, 1980). This phenomenon has been demonstrated experimentally in mice, where radiolabeled anti-paternal antigen antibodies were found to be taken up and digested by the placenta (Raghupathy et al., 1984; Wegmann et al., 1979; Wegmann et al., 1979). Unfortunately, experimental evidence difficult to obtain in human pregnancy. Thus, while the antibody barrier function of the placenta has been established in mice, its efficiency and significance for humans is controversial (Hunziker and Wegmann, 1986).

All trophoblast cells are also protected from complement-mediated destruction, a component of the innate immune system and a major effector mechanism of humoral immunity. These cells, including pre-implantation mural trophoblasts, express high levels of complement regulatory proteins on their surface (Holmes et al., 1992; Roberts et al., 1992; Vince and Johnson, 1995). Among these regulatory proteins are CD46 and CD59, both of which are involved in downregulating the classical and alternative complement cascades. Trophoblast CD46 was originally designated the trophoblast-leukocyte common antigen (TLX), a factor with known protective function (Purcell et al., 1990). Trophoblast expression of these regulatory proteins has been shown experimentally to provide protection from complement-mediated lysis (Tedesco et al., 1993).

The placenta is an antigen-bearing tissue that has developed unique mechanisms to protect itself from both humoral and cell-mediated immune responses. By expressing complement regulatory proteins, all populations of trophoblast cells are protected from complement-mediated lysis. In addition, by serving as an immunoabsorbent barrier between the maternal and fetal circulations, the placenta shields the fetus from anti-paternal humoral responses. With regards to cell-mediated immunity, invasive extravillous cytotrophoblasts may utilize HLA-G expression to mediate protective interactions with maternal leukocytes in the decidua. However, the mechanisms by which HLA–null villous syncytiotrophoblasts evade cellular immune attack are unknown.

Maternal immune response to pregnancy

In light of the apparent survival of the fetal allograft, the immunological competence of the mother has been questioned. Medawar first observed that Rh immunization, resulting in hemolytic disease in the fetus, occurs much less often than might be expected on "purely immunological grounds" (Medawar, 1954). However, a reason for its occurrence at all was not discussed, and seems to be proof that the mother's immune system is intact. Additional evidence comes from the observation that human mothers mount humoral immune responses against trophoblast antigens (Faulk and Hunt, 1989). Normal pregnant women appear to mount antibody responses against the complement-regulatory protein, TLX (McIntyre and Faulk, 1986). Antibodies against paternal HLA antigens have also been demonstrated to appear in the maternal circulation soon after implantation (Aksel, 1992; van Rood et al., 1958). In fact, this type of allosera was obtained from multigravidas and used for the initial discovery of the MHC. Maternal humoral immune responses may also be protective of the fetus. Some have suggested that pregnancy antisera contains a blocking activity that can suppress MHC-restricted immune reactions, although these antibodies have been poorly described (Power et al., 1983; Redman, 1990;

Rocklin et al., 1982). Others believe that women with chronic abortions lack this blocking antibody (Rocklin et al., 1976). Together, these observations suggest that maternal humoral responses are functional during pregnancy.

It has also been proposed that the maternal immune system is competent, yet systemically compromised during pregnancy (reviewed in Loke and King, 1991; Rocklin et al., 1979). Specifically, evidence suggests that cellular immune responses are suppressed, yet humoral reactions are unaffected, such as those described above. This hypothesis is supported by observations that pregnant women are at a greater risk for some infections, as well as cervical and breast carcinomas, each of which require cytotoxic lymphocyte activity for their elimination (reviewed in Beckerman, 1994; Weinberg, 1984). With regards to viral infections, Hepatitis A and B occur more commonly and with a more fulminant course in pregnancy (Borhanmanesh et al., 1973). African patients suffering from hepatitis suffer a 40% rate of coma with a 33% mortality during pregnancy, compared to an 8% rate of coma and no risk of mortality in nonpregnant controls (Beckerman, 1994). Susceptibility to protozoan diseases are also heightened in pregnancy: the symptoms of malaria are much more severe in pregnant women, including cerebral malaria, acute renal failure, disseminated intravascular coagulation, pulmonary edema, and splenic rupture (Weinberg, 1984). Even fungal infections pose a greater threat to pregnant women: coccidioidomycosis is the leading cause of maternal death in areas where it is endemic (Drutz and Catanzaro, 1978). Some intracellular bacterial infections are also more commonly seen in pregnant women. Pregnancy also increases a woman's risk for tuberculosis by 3-fold, as well as quickening the progression of leprosy. In addition, up to one third of all cases of listeriosis are found in pregnant women. Many of these microorganisms also have a special affinity for placental tissue (as discussed in the previous chapter), suggesting that local immune reactions are ineffective in their elimination.

With the recent development of antimicrobial therapy, many of these effects have been obscured in most populations, making these data difficult to confirm. Many believe that there are no differences in the numbers of circulating T cell subsets during pregnancy (Tallon et al., 1984). Some suggest that T cell function is unchanged during pregnancy (Gill and Repetti, 1979), while others report a decrease in lymphocyte proliferative responses in vitro (Gehrz et al., 1981). There is no apparent change in B cell counts and serum Ig levels during pregnancy, suggesting that humoral immunity is unaffected (Beckerman, 1994). Innate immunity is also intact, as there is no change in serum levels of complement components during pregnancy (Kovar and Riches, 1988). Therefore, the theory that pregnant woman are systemically immunosuppressed is highly controversial and may be result of subjective interpretations of anecdotal data.

The idea that the uterus is an immunologically privileged site, shielding the fetus from maternal immune rejection, has also been challenged (Billingham, 1964). Most importantly, this concept does not explain the apparent survival of rare human ectopic pregnancies occurring outside of the uterus (Martin et al., 1988). In addition, pregnancy is marked by a local inflammatory reaction at the implantation site (Marcus and Shelesnyak, 1968). Participating cells may include the large numbers of bone-marrow derived cells that infiltrate the decidualizing endometrium (Bulmer and Sunderland, 1983; Bulmer and Sunderland, 1984). Immunohistochemistry, coupled with quantitative characterization of decidual cells by flow cytometry, has identified three major populations of maternal immune effector cells, including T lymphocytes, macrophages, and large granular lymphocytes (LGLs).

Classical T lymphocytes bearing αß or γδ TCRs and expressing $CD4^+$ or $CD8^+$ are present in the human decidua (Kabawat et al., 1985). In fact, it has been shown that γδ T cell

subsets specifically home to the non-pregnant murine endometrium (Itohara et al., 1990). Most studies report an association of $CD8^+$ T cells with endometrial glands at all stages of pregnancy, while $CD4^+$ cells are more evenly distributed throughout the decidua (Mincheva-Nilsson et al., 1994). Some investigators have suggested a predominance of $CD4^+$ T cells, although this may be artifactual due to the expression of CD4 by decidual macrophages (Bulmer and Johnson, 1984). Attempts to characterize the relative percentages of αß or γδ T cells in the pregnant human uterus have yielded conflicting results. Some suggest that the population of γδ cells predominates in the decidua throughout pregnancy (Heyborne et al., 1992; Mincheva-Nilsson et al., 1992). Other studies utilizing different preparations of decidual cells from early pregnancy do not agree with this observation (Erbach et al., 1993; Morii et al., 1993).

The function of decidual γδ T cells has not be defined, but γδ cells are known to have cytotoxic activity (Moretta et al., 1991). In light of their strong reactivity to mycobacteria, this T cell subset may play an important role in uterine immunity against these pathogens (Chien et al., 1996). In addition, it appears that, unlike $CD8^+$ αß cells, γδ T cell cytotoxicity cannot be inhibited by supernatants from placental explant cultures (Menu et al., 1989). Others have shown that trophoblast cells are recognized by γδ T lymphocyte hybridomas in an MHC-independent manner (Heyborne et al., 1994). Together, these results suggest that trophoblasts are susceptible to lysis by γδ T cells, although the in vivo significance of these findings is unknown.

Macrophages are also present in the decidua, often directly adjacent to extravillous cytotrophoblasts (Goldstein et al., 1988; Hunt et al., 1984). Studies show that these macrophages express MHC class II antigens, suggesting that they are activated. These phagocytes may be important for the elimination microorganisms in cases of placental infection. Alternately, macrophages may present trophoblast antigens to decidual T cells to

stimulate an immune response. Macrophages are also involved in the infection of trophoblast cells with HIV, although their precise role has not been elucidated (reviewed in Douglas and King, 1992). Decidual macrophages have been shown to produce a wide variety of cytokines, and may be an integral part of the decidual cytokine network (Hunt and Pollard, 1992).

The third class of decidual leukocytes are the LGLs, named so because they are quite large and contain azurophilic granules within their cytoplasm (Bulmer and Sunderland, 1983). These cells are unique to the uterus, increasing in number during the luteal phase of the menstrual cycle and in early pregnancy (King et al., 1989). LGLs are CD4 and -8 negative, but express CD56, a marker for NK cells (Bulmer, 1989; Ritson and Bulmer, 1987). Their CD phenotype suggests that uterine LGLs are related to NK cells. To determine if these cells share effector functions, the ability of LGLs to kill standard NK targets was tested. Compared to NK cells, LGLs are relatively ineffective in lysing standard NK targets, but their killing can be upregulated by IL-2 stimulation (Ferry et al., 1990; Manaseki and Searle, 1989). In addition, LGLs display no cytotoxic activity against trophoblast cells, while IL-2 stimulated NK cells readily lyse these targets (King et al., 1989). While these cells seem to have limited cytotoxic activity, a potential function for LGLs in the uterus has not been described.

In addition to these well-characterized leukocyte subsets, some investigators have proposed the existence of a unique population of suppressor cells in the decidua. Daya et al. identified a trophoblast-depended suppressor cell type in the decidua, although its phenotype was not well defined (Daya et al., 1985). Others have demonstrated that crude preparations of decidual cells, the result of collagenase dispersion of whole decidual tissue, can have suppressive effects on lymphocyte alloreactivity in vitro (Parhar et al., 1988). Suppressor cells are thought to mediate protective immune interactions through the

secretion of immunoregulatory molecules (Daya et al., 1985; Daya et al., 1989). However, because this population is so poorly characterized, its existence is doubted by many (O'Hara, 1995). Instead, the immunoinhibitory effects once attributed to suppressor cells may be mediated by soluble factors produced by other, defined cell types.

Soluble mediators of immune function

Numerous immune functions are regulated by a wide variety of soluble factors, including molecules secreted by cells of the immune system as well as different cell types in the pregnant uterus. As an early student of transplant immunology, Medawar observed that "certain secretions of the adrenal cortex" (referring to corticosteroids) can inhibit allogeneic graft rejection in mice. He further suggested that these hormones are probably produced in excess during pregnancy, resulting in tolerance of the mother to the genetically foreign fetus. Since this was proposed, considerable work has focused on identifying soluble factors that have immunosuppressive activity at the fetomaternal interface.

Many studies have demonstrated that various preparations of placental cell and tissue types can suppress immune reactions in vitro. For example, the supernatants of first trimester trophoblast cell cultures have been shown to contain an undefined immunoregulatory activity (Sanyal et al., 1989). Whole organ cultures of first trimester chorionic villi also produce a soluble factor that inhibits T cell proliferation (Silver et al., 1990). Mitogen-induced T lymphocyte proliferation can be suppressed by the cytosolic fraction of homogenized whole term placenta (Remacle-Bonnet et al., 1983). Other unidentified molecules in the conditioned medium from cultures of a mixed population of placental cells inhibit both mitogen- and alloantigen-stimulated lymphocyte proliferation (Rubinstein et al., 1982). The ability of serum from pregnant patients to suppress alloreactive T cell proliferation suggests that the placenta secretes immunosuppressive factors in vivo, as well

(Arkwright et al., 1992). Although these effects were attributed to undefined immune mediators, a variety of molecules with known inhibitory activities have been shown to be produced by cells in the pregnant uterus (Table 2-1).

Among immunosuppressive factors at the fetomaternal interface, steroid hormones are present in high concentration locally. Progesterone can inhibit lymphocyte alloreactivity in mixed lymphocyte reactions (MLR), as well as downregulating mitogen-induced lymphocytes proliferation (Beer and Sio, 1982; Siiteri et al., 1977) Estrogen also suppresses T cell proliferative responses, but it may act to enhance humoral immunity (Siiteri and Stites, 1982; Sthoeger et al., 1988). In addition, hCG has been shown to both prolong skin allografts in mice, as well as inhibit lymphocyte reactivity to mitogens and alloantigens (Adcock et al., 1973). Inconsistent data from work with purified hCG suggests that there is a minimal role for this protein alone in the suppression of immune function in pregnancy (Muchmore and Blaese, 1977). The results from experiments testing the immunosuppressive activity of hPL have been similarly conflicting (Beer and Sio, 1982; Contractor and Davies, 1973).

Other placental factors with immunoinhibitory activity include prostaglandin E_2 (PGE_2), which is produced by maternal decidual macrophages in vitro (Norwitz et al., 1991). Prostaglandins have been shown to have immunosuppressive functions and may be important in dampening an inflammatory response in the decidua (Yagel et al., 1988). In addition to prostaglandins, an isomer of alpha-fetoprotein (AFP) can suppress lymphocyte proliferation, NK activity, and T-cell–dependent antibody secretion (van Oers et al., 1989). Pregnancy specific glycoproteins (PSGs), such as SP-1, can inhibit mitogen-as well as alloantigen-induced lymphocyte proliferation (Cerni et al., 1977). Pregnancy-associated plasma protein-A (PAPP-A) is another PSG that is produced by both human decidua and trophoblast, and can inhibit lymphocyte proliferation in vitro (Bischof et al., 1982).

Different cell types in the human placenta also secrete a variety of cytokines, molecules originally identified as potent regulators of the immune system. A comprehensive review of cytokine production in the pregnant uterus can be found in (Guilbert et al., 1993; Hunt, 1989; Pollard, 1991), and a partial list is shown in Table 2-2. These include both immunoinhibitory cytokines and those that function to stimulate immune responses. Immunosuppressive cytokines are thought to play a beneficial role in protecting allogeneic fetal cells from rejection, while inflammatory cytokines may be important in mediating the elimination of uterine infection. While both maternal and fetal cells in the decidua secrete cytokines, it is thought that the placenta plays an active role in secreting immunosuppressive cytokines to directly modulate maternal immunity. Some have proposed that placental cytokines specifically redirect the maternal immune system away from Th2–mediated cytotoxic responses, and towards Th-1, or humoral, immunity (Lin et al., 1993; Wegmann et al., 1993).

Clinical evidence suggests that pregnant women suffer from weakened cell-mediated immunity but heightened humoral responses. As discussed in the preceding section, there is substantial evidence that pregnant mothers are more susceptible to infections that require cytotoxic immune responses for their elimination. In addition, approximately 40% of women with the cell-mediated autoimmune disorder rheumatoid arthritis experience a temporary remission of their symptoms during pregnancy (Spector, 1990). Although these cytotoxic responses appear to be suppressed, humoral immunity may be strengthened, as systemic lupus erythematosis (SLE), a disease mediated by excessive autoantibody production, is often exacerbated in pregnant women (Varner, 1991). As with humans, murine pregnancy appears to lower the mothers' resistance to the intracellular pathogen *L. monocytogenes* (Luft and Remington, 1984). Because it has been demonstrated that Th1

cytokines are required for a curative immune response against *L. monocytogenes*, these observations suggest that pregnancy is a Th2 phenomenon.

Further experimental evidence comes from studies that demonstrate a predominance in Th2 cytokines produced by murine decidual and placental cells compared to maternal lymphoid tissue (Lin et al., 1993; Wegmann et al., 1993). Whether a Th2 cytokine bias occurs in human pregnancy is not known, however leukocytes from women with unexplained recurrent abortions produce Th1 cytokines in response to trophoblast antigens in vitro (Hill et al., 1995). In contrast, leukocytes from reproductively normal controls respond to trophoblast antigens by secreting Th2 cytokines. These observations suggest that recurrent abortions may be the result of an inappropriate maternal immune response to the placenta. Perhaps cytokines secreted by placental cells participate in the induction of a Th2 response, thus maintaining normal pregnancy.

In addition to the modulation of maternal immune response, fetal and maternal cytokines may regulate trophoblast differentiation to either limit or enhance the depth of uterine invasion. These effects could be mediated through the regulation of adhesion molecules or proteinases, as the correct expression of both are critical for cytotrophoblast differentiation in normal pregnancy (see Chapter 1). For example, TNF-α acts on endothelial cells to upregulate α1ß1 and downregulate α6ß1 expression (Defilippi et al., 1992). These responses are similar to those observed in differentiating invasive cytotrophoblasts (Damsky et al., 1992), suggesting that similar regulatory mechanisms may be important in the placental bed. Cytokines have also been shown to influence the expression and secretion of matrix metalloproteinases (MMPs) and their inhibitors in a variety of cell types (reviewed in Ries and Petrides, 1995). For example, IL-4 suppresses monocyte/macrophage secretion of MMP-9 to dampen their inflammatory activity (Lacraz et

al., 1992). Because MMP-9 is rate-limiting for cytotrophoblast invasion in vitro, its regulation by cytokines may affect cytotrophoblast invasion of the uterine wall.

An extensive list of soluble immunomodulatory factors have been shown to be produced by different cell types in the pregnancy uterus. This list includes cytokines, molecules known to influence the growth and differentiation of a variety of cell types, including cells of the immune system. Because cytokines are known to have autocrine and paracrine effects, it is difficult to ascertain the significance of their production by individual cells in gestational tissues. However, fetal and maternal cytokines are probably involved in the regulation of both placental immune protection and the control of cytotrophoblast invasion.

Conclusion

The fetus has historically been regarded as an allograft that is somehow protected from a harmful maternal immune response. Since biologists first proposed potential mechanisms for fetal evasion of maternal immunity, significant discoveries have been made in the field of reproductive immunology. It is now known that villus trophoblasts, which form the major population of trophoblast cells, do not express class I or II MHC antigens. The exception is invasive cytotrophoblasts, which express the nonclassical class I molecule, HLA-G. This suggests that the immunological relationship between a mother and her fetus is distinct from that between a host and a transplanted organ. Thus, unique mechanisms have evolved to allow maternal immune tolerance of her fetus, while largely maintaining her resistance to infectious organisms. Evidence implicates maternal and fetal cytokines in the regulation of these complex interactions. Among many cytokines' pleiotropic activities, the regulation of both adhesion molecule and proteinase expression may also affect trophoblast differentiation. This thesis (Chapters 3 and 4) will describe a role for cytotrophoblast-derived IL-10 in both of these processes. Its activities include the

INNATE
SPECIFIC
cell-mediated
humoral
T cells
B cell
Th
(cytokines)
(Ts?)
Th2
Th1
CTL
plamsa cell
Igs
opsonization
physical barriers
natural killer cells
mononuclear phagocytes
(DTH)
complement

Figure 2-1

Figure 2-2: Diagram of class I and II major histocompatibility molecules. Class I molecules are composed of a polymorphic α chain non-covalently bound to the invariant ß chain, ß2microglobulin. Class II molecules consist of two polymorphic α chains. (Modified from Abbas 1994, with permission).

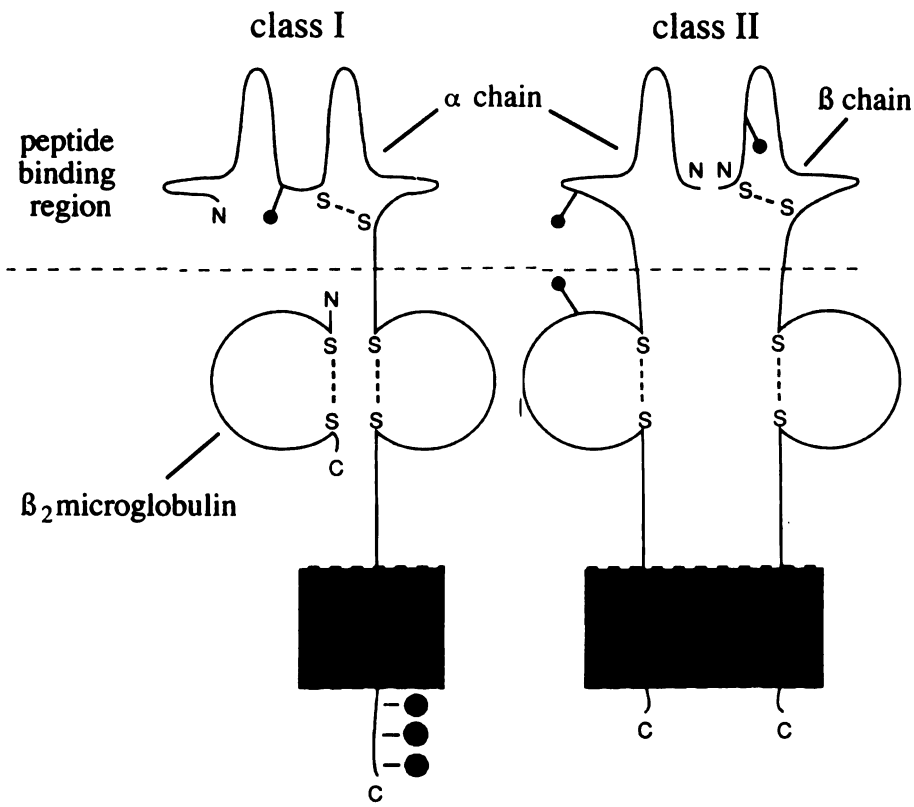

class I
class II
α chain
ß chain
peptide
binding
region
N
S
S
N
N
S
S
N
S
S
S
S
S
S
S
S
C
ß2microglobulin
C
C
C

Figure 2-2

Figure 2-3: The T cell receptor complex. The TCR is composed of α and ß, or γ and δ chain subunits. The TCR is additionally noncovalently associated with up to five other membrane proteins, including CD3 (γ, δ, and ε chains) and either a homodimer of ζ chains, or a ζη heterodimer. (Modified from Abbas 1994, with permission).

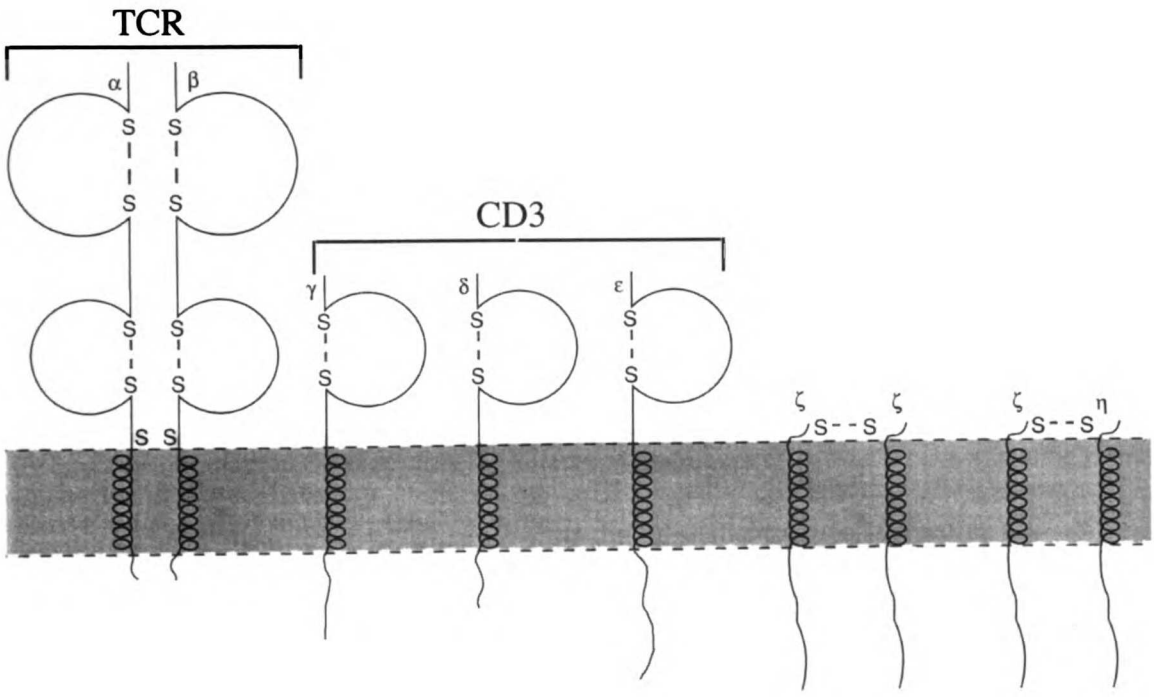


Figure 2-3

Figure 2-4: T cell antigen recognition and stimulation. Foreign peptides are presented to $CD8^+$ cytotoxic T lymphocytes (CTL) by class I MHC molecules on target cells, while class II molecules on antigen presenting cells (APC) present peptides to $CD4^+$ T helper (Th) cells. Ligand binding by several T cell accessory molecules is required for activation of an immune response. (Modified from Abbas 1994, with permission).

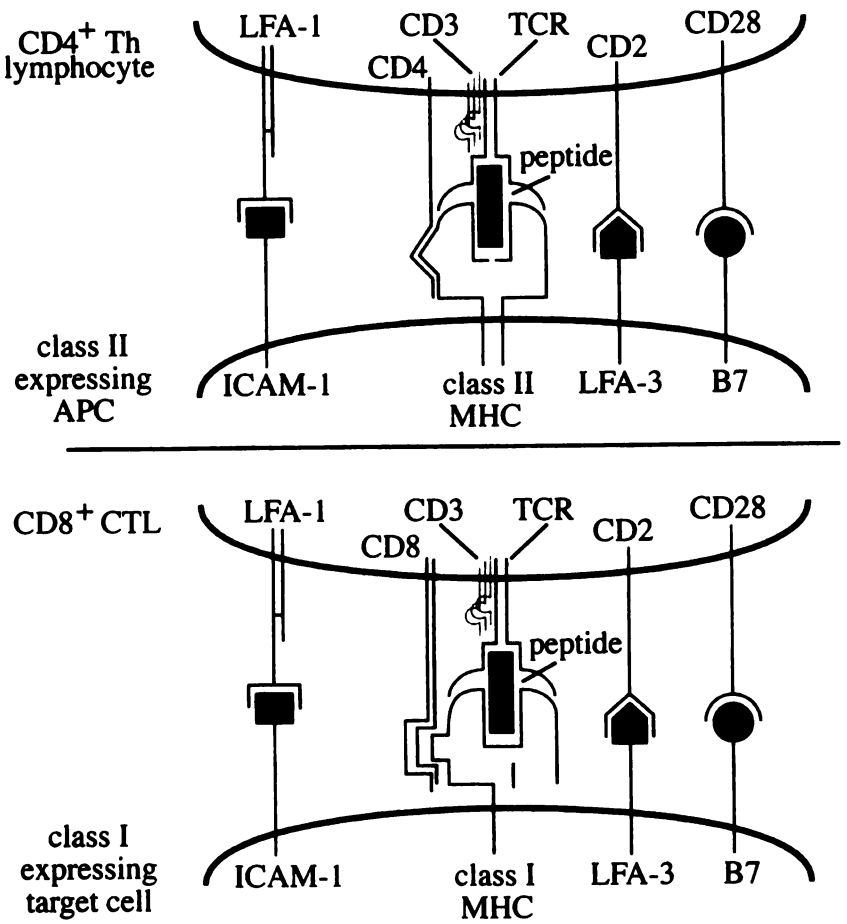

CD4+ Th lymphocyte
LFA-1
CD3
TCR
CD2
CD28
CD4
peptide
class II expressing APC
ICAM-1
class II MHC
LFA-3
B7
CD8+ CTL
LFA-1
CD3
TCR
CD2
CD28
CD8
peptide
class I expressing target cell
ICAM-1
class I MHC
LFA-3
B7

Figure 2-4

Table 2-1. Immunosuppressive factors produced by cells at the maternal-fetal interface in human pregnancy

Undefined factors (source)	Reference
CTB/STB CM	(Sanyal et al., 1989)
chorionic villi CM	(Silver et al., 1990)
term placenta homogenate	(Remacle-Bonnet et al., 1983)
dispersed placental cells	(Rubinstein et al., 1982)
maternal serum	(Arkwright et al., 1992)

Defined factors	Reference
progesterone	(Beer and Sio, 1982; Siiteri et al., 1977)
hPL	(Beer and Sio, 1982; Contractor and Davies, 1973)
prolactin	(Beer and Sio, 1982)
estrogens	(Siiteri and Stites, 1982)
SP-1	(Cerni et al., 1977)
PAPP-A	(Bischof et al., 1982)
AFP	(van Oers et al., 1989)
hCG	(Adcock et al., 1973; Muchmore and Blaese, 1977)
PGE_2	(Norwitz et al., 1991; Yagel et al., 1988)

Table 2-2. Cytokines produced by cells at the maternal-fetal interface in human pregnancy.

Cytokine	Source	Possible function	Reference
IL-1	CTB, STB, X, fetal macrophages, maternal macrophages	Cytokine synthesis, steroidogenesis, regulation of cytotrophoblast invasion	(Flynn et al., 1982) (Kauma et al., 1990) (Librach et al., 1994) (Nestler, 1993)
IL-2	STB	?	(Boehm et al., 1989)
IL-6	STB	Hormone synthesis	(Nishino et al., 1990) (Kameda et al., 1990) (Stephanou and Handwerger, 1994)
IL-8	CTB, STB, Hofbauer	?	(Saito et al., 1994) (Stephanou and Handwerger, 1994)
IL-10	CTB	immunosuppression regulation of CTB invasion	(Roth et al., 1996)
IL-15	placental cDNA	stimulate humoral immunity	(Armitage et al., 1995)
IFN-α, β, γ	placental explants	?	(Tao and Cao, 1993)
TGF-ß2	decidual leukocytes	immunosuppression	(Christmas et al., 1990)
TGF-ß1	decidua, placenta	immunosuppression	(Kauma et al., 1990) (Dungy et al., 1991)
TNF-a	decidual macrophage	?	(Vince et al., 1992)
CSF-1	decidua PFib	stimulate trophoblast proliferation CTB syncytialization	(Jokhi et al., 1995) (Garcia-Lloret et al., 1994)
G-CSF	CTB, STB, decidua	?	(Saito et al., 1994) (McCracken et al., 1996)
GM-CSF	PFib	CTB syncytialization	(Garcia-Lloret et al., 1994)
M-CSF	placenta decidua	?	(Daiter et al., 1992) (Kanzaki et al., 1992)

CHAPTER THREE

Immune Suppression by Human Placental Cytotrophoblast IL-10

Abstract

The mechanism by which the mammalian mother accepts the implanting fetus as an allograft remains unexplained, but is likely to be the result of a combination of factors. Mononuclear cytotrophoblasts, the specialized fetal cells of the placenta that invade the uterus, play an important role. These cells express HLA-G, an unusual MHC class I-B molecule, and secrete cytokines and pregnancy-specific proteins that can regulate immune function. We investigated whether cytotrophoblasts secrete interleukin 10 (IL-10), a cytokine that potently inhibits alloresponses in mixed lymphocyte reactions. Cytotrophoblasts from all stages of pregnancy produced IL-10 in vitro, but neither placental fibroblasts nor choriocarcinoma (malignant trophoblast) cell lines did so. Spontaneous IL-10 production averaged 650, 853 and 992 pg/10^6 cells in the first, second and third trimesters of pregnancy, respectively. IL-10 secretion dropped approximately 10-fold after the first 24 h of culture and was paralleled by a decrease in mRNA. IL-10 mRNA was detected in biopsies of the placenta and the portion of the uterus which contains invasive cytotrophoblasts, suggesting that this cytokine is also produced in vivo. IL-10 secreted by cytotrophoblasts in vitro is bioactive as determined by its ability to suppress IFN-γ production in an allogeneic mixed lymphocyte reaction. We conclude that human cytotrophoblast IL-10 may be an important factor that contributes to maternal tolerance of the allogeneic fetus.

Introduction

During human pregnancy, genetically foreign cells from the fetal portion of the placenta invade the uterus. This process is the result of differentiation of the specialized epithelial cells of the placenta, termed trophoblasts (reviewed in Damsky et al., 1993 and Cross et al., 1994). Mononuclear cytotrophoblast stem cells are anchored to basement membranes surrounding the stromal cores of two types of chorionic villi. In floating villi, cytotrophoblasts fuse to form the overlying syncytium which is in direct contact with maternal blood, mediating nutrient and gas exchange for the developing fetus. In anchoring villi, cytotrophoblasts differentiate by leaving their basement membrane and forming columns of cells. These cell columns give rise to the invasive subpopulation of cytotrophoblasts that attaches to and invades the uterus and its arterial system. In response to cytotrophoblast invasion, the uterine stroma decidualizes and is infiltrated by a specific subset of maternal immune cells. As a result, the pregnant human uterus contains a mixture of fetal and maternal cells, the latter being up to 75% immune-competent bone marrow-derived cells (Starkey et al., 1988).

Because of their unique position at the maternal-fetal interface, placental trophoblast cells are presumed to shield the allogeneic fetus from rejection (Beer and Sio, 1982; Sionov et al., 1993), although the molecular mechanisms have not been fully defined. Faulk et al. (Faulk et al., 1977; Faulk and Temple, 1976) suggested that the placenta passively evades immune recognition through the absence of classical class I and II MHC antigens on syncytiotrophoblast cells. However, subsequent studies revealed that invasive cytotrophoblasts express a nonclassical class I antigen, HLA-G (Ellis et al., 1986; Kovats et al., 1990; McMaster et al., 1995). It has been suggested that this molecule could function as a universal "self" transplantation antigen preventing maternal immune attack of the feto-placental unit (Kovats et al., 1990; Redman, 1990).

The localized secretion of immunomodulatory factors by the placenta may also contribute to survival of the fetal allograft. Hormones in high concentration at the placental-uterine interface, such as progesterone, human placental lactogen, prolactin, and estrogens, inhibit lymphocyte reactivity in vitro (Siiteri and Stites, 1982). Other placental factors with inhibitory effects on immune function include pregnancy-associated α-2-glycoprotein, pregnancy-associated plasma protein-A, and α-fetoprotein (Rocklin et al., 1979). In addition to peptide and steroid hormones, the human placenta secretes a variety of cytokines, including CSF-1 (Kauma et al., 1991), IL-1ß (Librach et al., 1994), IL-6 (Kameda et al., 1990; Nishino et al., 1990) and TGF-ß (Dungy et al., 1991; Kauma et al., 1990).

Alloreactive T-lymphocyte responses are also suppressed by other, as yet undefined substances in cytotrophoblast conditioned medium (Silver et al., 1990) and in the supernatants from co-cultures of cyto- and syncytiotrophoblasts (Sanyal et al., 1989). This immunosuppressive activity is very similar to the biological effects described for IL-10, an immunoregulatory cytokine that inhibits the generation of alloreactive T cells in mixed lymphocyte reactions (MLR; Bejarano et al., 1992). To test the possibility that IL-10 may be involved in trophoblast-mediated maternal immune inhibition, we investigated the production of IL-10 by several types of human placental cells, including primary cyto- and syncytiotrophoblasts, placental fibroblasts, and choriocarcinoma cell lines. The results of this study show that IL-10 is produced specifically by primary cytotrophoblasts in vitro and that this cytokine can inhibit an allogeneic immune response. We hypothesize that IL-10 may play an important role in suppressing potentially harmful maternal immune responses in vivo.

Results

Human placental cytotrophoblasts secrete IL-10 in vitro. We first examined cytokine synthesis by a variety of placental cells, using ELISA. To test cytotrophoblast production of cytokines, we utilized an in vitro system shown by stage-specific antigen expression (reviewed in Damsky et al., 1993 and Cross et al., 1994) to model the differentiation of these cells along the invasive pathway in vivo. To obtain the required number of cells, first trimester cytotrophoblasts were isolated from 5-10 pooled placentas, second trimester cells from 1-4 pooled placentas, and term cells from a single placenta. Cytotrophoblast populations prepared in this way are 95% free of CD-45–positive immune cells, themselves potential cytokine-producing contaminants (Librach et al., 1991). We also analyzed conditioned medium from organ cultures of intact chorionic villi. Under these conditions, syncytiotrophoblasts are the primary source of secreted molecules. Cytokine production by other placental cell types, including first trimester placental fibroblasts and the BeWo, JAR and JEG choriocarcinoma cells lines, was also investigated.

We recently showed that purified cytotrophoblasts produce IL-1ß, with highest levels secreted during the first trimester of pregnancy (Librach et al., 1994). In the present experiments we confirmed previous reports that these cells produce IL-6, but neither IL-2, -3, -4, -5, -7 nor IFN-γ were detected by ELISA in conditioned media (data not shown). In contrast, all cultures contained IL-10 in levels that were greater than 50 pg/ml, the minimum sensitivity of the assay (Fig. 3-1). There were no significant differences in the mean IL-10 levels produced by cytotrophoblasts from different gestational ages. However, there were substantial differences in the absolute amount of IL-10 secreted by cells obtained from different pregnancies. Human immune cells secrete IL-10 in vitro after activation with agents such as endotoxin (de Waal Malefyt et al., 1991). Although cytotrophoblasts secreted IL-10 without stimulation, addition of 1 µg/ml LPS to first trimester cells

increased IL-10 secretion approximately 3-fold compared to untreated cells from the same preparation. With regard to other placental cell types, 0.1 g of chorionic villi produced approximately 2 ng IL-6, but less than 50 pg of IL-10, suggesting that syncytiotrophoblasts do not produce considerable quantities of the latter cytokine. In addition, neither the conditioned media nor lysates of placental fibroblasts or choriocarcinoma cells contained IL-10. These data suggest that cytotrophoblasts are the major placental source of IL-10 in vitro and that the choriocarcinoma cell lines do not secrete this cytokine.

We used a second experimental approach to verify that human cytotrophoblasts synthesize IL-10. Purified cells were cultured for 12 h and immunostained with mAbs specific for IL-10. A mAb to IL-7 that was matched for IgG subtype was used as a control. Purified cytotrophoblasts form aggregates during the first 48 h of culture when plated at subconfluent levels (Librach et al., 1991). A subset of such cells reacted with IL-10 antibodies Fig. 3-2*a*) but not with IL-7 antibodies (Fig. 3-2*b*). In accordance with the ELISA data, placental fibroblasts and the choriocarcinoma cell lines did not stain positively for IL-10.

Temporal regulation of IL-10 production. To establish that cytotrophoblasts produce, as well as secrete IL-10 in vitro, we immunoprecipitated this protein from lysates and conditioned media prepared from metabolically radiolabeled cells (Fig. 3-3). Autoradiography of the electrophoretically separated immunoprecipitates showed a single band corresponding to the molecular mass of human IL-10 (18 kD). This band was most prominent in conditioned media collected from cells that had been cultured for 12 h, and it decreased in intensity over the 60-h culture period. Cell lysates contained much lower, but still detectable levels of IL-10, and again the highest amount was seen after 12 h in culture. No specific bands were detected in immunoprecipitates obtained using an antibody to IL-7

(25 kD). These results suggest that cytotrophoblast synthesis of IL-10 is highest during the first 12 h, and that this cytokine is rapidly secreted into the medium. This was verified by ELISA, which showed that the amount of IL-10 secreted by cytotrophoblasts dropped after 24 h to levels that were below the sensitivity of the assay. During this same period, cytotrophoblast cells cultured under identical conditions remain viable as indicated by their continued secretion of placental hormones (e.g., human chorionic gonadotropin; (Fisher et al., 1989)) and by their expression of newly synthesized stage-specific antigens (reviewed in Damsky et al., 1993 and Cross et al., 1994).

Mechanism of downregulation of IL-10 synthesis. To elucidate the mechanism by which protein synthesis is downregulated, we used a competitive PCR-based method with primers specific for human IL-10 to measure mRNA levels in cultured cells. We were unable to detect message for this cytokine in samples from control cultures of placental fibroblasts or the BeWo, JAR, and JEG cell lines. IL-10 message was, however, readily detected in first, second, and third trimester cytotrophoblasts (Fig. 3-4) both at the time of isolation (time 0) and after as long as 2 days in vitro (48 h). In accordance with the immunoprecipitation and ELISA data, the amount of IL-10 mRNA (as normalized to HPRT) decreased with time in culture. Together, these data suggest that the decline in IL-10 production in vitro is transcriptionally regulated.

The presence of IL-10 mRNA in cytotrophoblasts immediately after purification suggests that these cells either synthesize IL-10 in vivo or are induced to do so by the isolation procedure. To begin to address this question, we used RT-PCR to analyze various tissues that are components of the maternal-fetal interface. Both chorionic villi and the portion of the uterus to which anchoring villi attach expressed IL-10 mRNA (data not shown). Since these specimens contain a variety of cell types in addition to cytotrophoblasts, it remains to be determined which of these cells produce IL-10 in vivo.

Cytotrophoblast IL-10 is immunoinhibitory. Next, we investigated whether it was possible to detect one biological activity of IL-10, namely the ability to inhibit an allogeneic immune response, within the complex mixture of growth factors and cytokines that cytotrophoblasts secrete. Specifically, we measured the ability of cytotrophoblast conditioned medium to inhibit IFN-γ production in a MLR. In control experiments, the addition of 1-100 U rIL-10 to a MLR inhibited IFN-γ secretion in a dose-dependent manner. As previously shown, as little as 1U rIL-10 was sufficient to reduce IFN-γ levels by approximately 25% and the function-perturbing antibody 19F1 reversed this suppressive activity (Bejarano et al., 1992). In addition, 19F1 increased basal IFN-γ levels by neutralizing endogenous IL-10 when added alone to a MLR. These effects were not observed when a nonspecific antibody of the same IgG subtype was used as a control. Addition of conditioned media from different preparations of cytotrophoblasts to a MLR had variable effects on IFN-γ production (Fig. 3-5). However, neutralizing IL-10 in cytotrophoblast conditioned media resulted in a significant increase in IFN-γ secretion in many samples ($p < 0.05$). In these experiments, the ability to stimulate IFN-γ production positively correlated with the amount of IL-10 in the conditioned medium. Titrating 19F1 in the conditioned medium of one preparation of cytotrophoblasts resulted in a dose-response stimulation of IFN-γ production (Fig. 3-6a). Again, the isotype-matched control antibody had no effect (Fig. 3-6b). These observations show that IL-10 secreted by cytotrophoblasts acts to inhibit allogeneic lymphocyte reactivity in vitro.

Discussion

We report that IL-10 secreted by highly purified human placental cytotrophoblasts can suppress an allogeneic immune response in vitro. Human IL-10 is a pleiotropic cytokine that is produced by activated immune cells including human $CD4^+$ T-cell clones (Th0, Th1, and Th2 cells; Moore et al., 1993), B cells (Matthes et al., 1993), and monocytes (de Waal Malefyt et al., 1991), by UV-irradiated murine keratinocytes (Rivas and Ullrich, 1992) and by a variety of human carcinoma cell lines (Gastl et al., 1993). Among its activities, IL-10 suppresses both T cell proliferative responses and IFN-γ production in MLRs when allogeneic cells are used as stimulators (Bejarano et al., 1992). IL-10 suppresses alloresponses by inhibiting the expression of both MHC class II molecules and the costimulatory molecule B7 (de Waal Malefyt et al., 1991; Ding et al., 1993). Alloreactive T cells, such as those generated in these reactions, play a key role in allograft rejection (Ferrara and Deeg, 1991). These effects of IL-10 make it an attractive candidate for suppressing transplant rejection and prolonging graft survival (Howard and O'Garra, 1992).

To investigate the possibility that IL-10 may be involved in protecting the allogeneic fetal "transplant" in human pregnancy, we determined whether placental cells synthesize this cytokine. Of those we studied, only primary cytotrophoblasts secreted IL-10 in vitro, with levels ranging from 67 to 2190 pg/ml during the first 24 h of culture. The systemic production of IL-10 in different human diseases is equally inconsistent. Serum levels of IL-10 in patients with meningococcal septic shock (Lehmann et al., 1995) or diffuse large cell lymphoma (Cortes et al., 1995) range from 25 to 64,500 pg/ml and $\leq$ 5 to 27,143 pg/ml, respectively. Even a clonal population of mice injected with an antibody to CD3 display a 9-fold variation in serum concentrations of IL-10 (Durez et al., 1993). The wide dispersion of IL-10 production by cytotrophoblasts is also reflective of the large biologic

variation in pregnancy-specific hormone production by different placentas. For example, the amount of human placental lactogen (hPL) released by cultured placentas covers a 5-fold range of values (Suwa and Friesen, 1969). This variability is also observed in vivo, where circulating levels of hPL in normal pregnancy range from 3.3 μg/ml to 25 μg/ml in the third trimester (DeGroot, 1989). As each placenta is exposed to a unique immune and hormonal environment in utero, it follows that the production of IL-10 will be regulated differently in each pregnancy.

Purified cytotrophoblasts contain IL-10 mRNA immediately after the isolation procedure, before culture. Both IL-10 gene transcription and subsequent protein secretion drop within 24 h in vitro. During this time, cytotrophoblasts are actively differentiating as they do during uterine invasion in vivo; they modulate the expression of molecules whose functions mediate either maternal immune tolerence (e.g., HLA-G; McMaster et al., 1995) or invasion (e.g., integrin cell-ECM receptors [Damsky et al., 1994] and MMP-9 [Fisher et al., 1989]). We have evidence that IL-10 can affect both of these important functions. With regard to immune aspects of pregnancy, we show here that IL-10 in cytotrophoblast conditioned media inhibits alloreactivity. In addition, preliminary data suggest that endogenous IL-10 upregulates the production of MMP-9, required for invasion in vitro (Librach et al., 1991).

It has been suggested that the maternal immune system in pregnancy is biased towards antibody production, while harmful cell-mediated immunity is weakened (Wegmann et al., 1993). The generation of a humoral response is associated with the production of Th2-type cytokines, including IL-3, IL-4, IL-5, IL-10, and IL-13. Of these, IL-10 directly inhibits synthesis of proinflammatory Th1-type cytokines (e.g. IFN-γ), thus preventing the development of cytotoxic immune cells (Fiorentino et al., 1989; Moore et al., 1993). Several groups have demonstrated the production of Th2 cytokines by tissues at the

maternal–fetal interface in murine gestation (Delassus et al., 1994; Lin et al., 1993). However, the precise identity of the cell types producing these cytokines has not been elucidated. This report shows that in human pregnancy, cytotrophoblasts secrete IL-10. Consistent with the ability of IL-10 to inhibit the production of Th1 cytokines, we did not detect IFN-γ in purified cytotrophoblasts by any of several methods used (Roberts, 1991). We postulate that the localized production of IL-10 by the placenta serves to protect the human fetus by driving the maternal immune system away from a potentially deleterious cell-mediated Th1 response.

For IL-10 to function as a local immunoinhibitor in pregnancy, its levels within the uterine wall must be sufficient to suppress a harmful maternal immune response. Since 100 pg/ml recombinant IL-10 is sufficient to inhibit T-cell proliferation in a MLR (Bejarano et al., 1992), the amount of IL-10 secreted by these cells in vitro is in a physiologically relevant range. In addition, we and others have detected IL-10 mRNA in biopsies of both placenta (Cadet et al., 1995) and placental bed, the decidualized uterine tissue into which cytotrophoblasts invade. IL-10 has also been demonstrated to be present in human amniotic fluid (Heyborne et al., 1994). Together, these observations suggest that this cytokine could be produced in sufficient amounts to be active in vivo. We have also shown that cytotrophoblast-derived IL-10 is immunosuppressive in vitro, as determined by its effect on IFN-γ secretion in a MLR. The fact that we detected this activity in the complex growth factor and cytokine mixture in conditioned medium suggests that IL-10 could play an important role in maintaining the fetal allograft.

The significance of placental IL-10 production in vivo is difficult to assess in animal models because many aspects of human pregnancy are unique. For example, human mononuclear cytotrophoblasts behave like tumor cells in that they migrate deep into the uterine wall, mixing extensively with maternal decidual cells (Cross et al., 1994). Mouse

placentation is characterized by shallow invasion by multinucleate giant cells which results in the formation of a distinct fetal-maternal boundary (Cross et al., 1994). Human pregnancy is also immunologically unique as evidenced by the fact that invasive cytotrophoblasts express HLA-G (Ellis et al., 1986; Kovats et al., 1990; McMaster et al., 1995) Whether there is a mouse HLA-G homolog is not known. In addition, the substantial difference in the length of gestation in the two species (20 days in the mouse vs 40 weeks in the human) suggests that different immunmodulatory mechanisms might be required for protection of the allogeneic fetus. Therefore, the successful birth of IL-10 deficient mice (Kuhn et al., 1993) does not exclude the possibility that this cytokine is important for the maintenance of human pregnancy. In fact, recent evidence suggests that IL-10 production is disregulated in some reproductive pathologies. IL-10 levels are significantly elevated in the amniotic fluid of small-for-gestational-age pregnancies, these being the leading cause of perinatal morbidity and mortality (Heyborne et al., 1994). Additionally, PBMCs isolated from women suffering from recurrent spontaneous abortions were found to secrete the Th1 cytokines INF-γ, IL-2, TNF-β, and TNF-α when stimulated with trophoblast antigens in vitro (Hill et al., 1995). In contrast, the supernatant of PBMCs from reproductively normal women contained IL-10, with undetectable levels of Th1 cytokines. These observations of human pregnancy suggest that IL-10 may be an important factor for a healthy, term gestation.

The observed maternal immune tolerance of the genetically foreign fetus in mammalian pregnancy is a complex phenomenon. Undoubtedly, multiple mechanisms are involved in preventing immune rejection of the feto-placental unit. The expression of HLA-G by invasive cytotrophoblasts is one way by which the human placenta can shield the conceptus from an immune attack. In addition to this evasive mechanism, the localized secretion of immunoregulatory cytokines may be very important in dampening an inflammatory immune

Methods

Placental Cell and Tissue Isolation and Culture. Cytotrophoblasts were isolated from first, second (Fisher et al., 1989; Librach et al., 1991), and third trimester (Kliman et al., 1986) human placentas by published methods. Chorionic villi were collected immediately after vacuum aspiration and washed three times in wash medium (DME H-21, 2.5% FCS, 1% penicillin/streptomycin). Placental fibroblasts were isolated from first trimester placentas as previously described (Fisher et al., 1989). The JAR (Pattillo, 1971), BeWo (Pattillo and Gey, 1968) and JEG (Kohler and Bridson, 1971) human choriocarcinoma cell lines (American Type Culture Collection, Rockville, MD) were maintained as previously described (Fisher et al., 1989).

Cytotrophoblasts, choriocarcinoma cell lines, and fibroblasts ($1x10^6$) and chorionic villi (0.1 g) were cultured in 1 ml of serum-free medium (Dulbecco's Modified Eagle's H21 minimal essential medium containing 2% Nutridoma [Boehringer Mannheim, Indianapolis, IN] and 50 µg/ml gentimicin; SFM) in 16-mm culture wells precoated with a basement membrane substrate (Matrigel; Collaborative Research Inc., Bedford, MA) diluted two parts to one part SFM as described (Librach et al., 1991). For immunohistochemical studies, cells were plated at a density of 2.5×10^5 cells/ 500 µl SFM in each well of an 8-well chamber slide (Nunc, Inc., Naperville, IL). PBMC were cultured in RPMI 1640 supplemented with 10% heat-inactivated human AB serum.

Where specified, IL-10 production was stimulated by the addition of 1 µg/ml LPS from *Escherichia coli* (Serotype No. 0127:B8; Sigma) to SFM. For metabolic labeling, 1×10^6 cytotrophoblasts were cultured for 12 h in 1 ml cysteine- and methionine-deficient DME containing 500 µCi Tran^{35}S-label™ (ICN Biomedicals Inc., Costa Mesa, CA).

Quantification of IL-10 in Conditioned Medium and Cell Extracts. Conditioned medium (CM) was collected after 24 h and centrifuged at 300 *g* for 8 min. CM was then concentrated using Centriprep (5 to 15 ml medium) or Centricon (up to 3 ml medium) concentrators with a 10,000 mol wt cutoff (Amicon, Inc., Beverly, MA). Cell extracts were prepared by adding 1 ml lysis buffer (0.5% NP-40, 150 mM NaCl, 25 mM Tris-HCl, pH 7.5) containing 1 mM PMSF to the cultures. The resulting suspensions were passed 10 times through a 26-gauge needle. Lysates were then centrifuged at 16,000 *g* for 5 min to remove cellular debris and filtered through a 0.2-μm filter. Conditioned media and lysates were stored at -80°C. IL-10 levels were determined by an ELISA developed in this laboratory as previously described (Abrams, 1995; Abrams et al., 1992; de Waal Malefyt et al., 1991). The sensitivity of the assay is 50 pg/ml.

Immunocytochemistry. Cells cultured overnight in chamber slides were fixed for 15 min in acetone at -20°C, then incubated in methanol containing 0.3% H_2O_2 for 10 min to block endogenous peroxidase activity. After a 1-h incubation in antibody diluent (PBS containing 0.7% gelatin from cold-water fish skin; Sigma), a mixture of 25 μg/ml each of two mAbs to human IL-10 (19F1 [Andersson et al., 1992] and 12G8 [Abrams et al., 1992; Andersson et al., 1992]) was added for 48 h at 4°C. We were unable to detect IL-7 in cytotrophoblast conditioned medium by ELISA (data not shown). Consequently, as a negative control, cytotrophoblasts were exposed to 50 μg/ml of a rat mAb to human IL-7 (BVD10-11C10; Andersson et al., 1992) that was of the same IgG subclass as both 19F1 and 12G8. As an additional control, cells were exposed to antibody diluent alone. Slides were incubated with a 1:100 dilution of biotinylated goat anti–rat IgG (Vector Laboratories, Inc., Burlingame, CA) for 60 min at room temperature. ABC reagent solution (Vector) was then added for 60 min, and the peroxidase reaction was developed by incubating with 3, 3'-diaminobenzidine (Vector) containing NiCl. Stained cytotrophoblasts were examined

by bright field optics with a Zeiss Axiophot microscope and photographed with Kodachrome 160 ASA tungsten film.

Immunoprecipitation. 1 x 10^6 metabolically labeled cells were extracted in 1 ml 2.5% Triton dilution buffer (TDB; Casanova et al., 1991) containing 5 µg/ml pepstatin, 10 µg/ml chymostatin, 5 µg/ml leupeptin, 10 µg/ml antipain, 0.5 nM benzamidin, 0.5 U/ml trasylol, and 1 mM PMSF. Conditioned medium (1 ml) was added to 0.5 ml TDB, and both medium and labeled cells were frozen at –80°C prior to use (1-7 days). Lysates and conditioned media were precleared twice with CL2-B beads (Pharmacia LKB Biotechnology, Piscataway, NJ) as described (Breitfeld et al., 1989). As an additional preclearing step, supernatants were added to 50 µl of a 30% (v/v) slurry of protein A-Sepharose (PAS) beads in PBS containing 0.03% NaN_3 and incubated with a rat IgG_{2a} mAb to ß-Galactosidase (GL117, 10 µg/ml) for 6 h at 4°C with continuous agitation. After 30 min, rabbit anti-rat IgG (10 µg/ml, Vector) was added to each tube. The precleared samples were then incubated with PAS and 3.3 µg/ml each of three mAbs to human IL-10 (12G8, 9D7 [Abrams et al., 1992] and 19F1 [Andersson et al., 1992]) with constant agitation for 12 h at 4°C. Rabbit anti-rat IgG (10 µg/ml, Vector) was added after 30 min. PAS beads were then pelleted by centrifugation and washed 5 times with mixed micelle buffer and 3 times with final wash buffer (FWB; Breitfeld et al., 1989), with a transfer of the beads to clean tubes during the second FWB wash. Beads were boiled for 3 min in Laemmli sample buffer containing 10% ß-mercaptoethanol and subjected to SDS-PAGE on a 15% acrylamide gel. The gel was fixed for 10 min in glacial acetic acid, incubated for 1 h in EN^3HANCE (DuPont Co., Wilmington, DE), rinsed twice in tap water and incubated in ddH_2O containing 3% glycerol for 1 h to precipitate the fluor. The gel was then dried and exposed to X-OMAT AR film (Kodak).

Analysis of Cytotrophoblast IL-10 mRNA. mRNA was extracted from 10^6 cells using RNAzol™ B (Biotecx Laboratories, Inc., Houston, TX) according to the manufacturer's recommendations. RNA was reverse transcribed with murine Moloney leukemia virus reverse transcriptase (GIBCO BRL, Bethesda, MD) and random hexamer primers (Sigma) as previously described (Corry et al., 1996; Reiner et al., 1993). To quantitate levels of IL-10 mRNA, cDNA was amplified in the presence of a multiple human cytokine competitor plasmid otherwise identical to a murine construct described in detail elsewhere (Corry et al., 1996; Reiner et al., 1993). Briefly, the competitor plasmid, called DC10, consists of tandemly arrayed authentic target sequences into which DNA spacers of 75-150 bp were introduced. Target sequences in the competitor construct amplify with equivalent efficiency in the presence of cDNA and are easily distinguished from the smaller wild-type amplimers by 2% agarose gel electrophoresis and ethidium bromide staining. A competitor for the constituitively-expressed gene, hypoxanthine-guanine phosphoribosyltransferase (HPRT)[1], was included in DC10, allowing the standardization of input cDNA to comparable levels of HPRT expression in each sample. Amounts of cDNA in each reaction were equalized by adjusting the amounts of input cDNA while maintaining a constant concentration of DC10 until the ratio of band intensities between competitor and wild-type amplimers were identical in each sample. These amounts of cDNA were then used for amplification of IL-10 followed by electrophoresis and ethidium bromide staining. Competition for reagents between the competitor, whose concentration remained constant, and varying amounts of IL-10 cDNA in each reaction resulted in different ratios of band intensities. As cDNA levels decreased, the larger competitor construct was preferentially amplified, resulting in a brighter band. These relative amounts of wild-type and competitor reaction products provide a semi-quantitative determination of relative levels of IL-10 mRNA in each sample. Primers sequences were as follows: HPRT 3': CCTGCTGGATTACATCAAAGCACTG; HPRT 5': TCCAACACTTCGTGGGGTCCT;

IL-10 3': ATGCCCCAAGCTGAGAACCAAGACCCAGAC; IL-10 5': TCTCAAGGGGCTGGGTCAGCTATCCCA.

PCR of placental biopsies: Total RNA was isolated from frozen, pulverized biopsies of term placenta and placental bed by using RNAzol™ B (Biotecx Laboratories, Inc., Houston, TX) according to the manufacturer's recommendations and reverse transcribed as described above. IL-10 cDNA was amplified using primers shown above.

Mixed Lymphocyte Reaction (MLR). Periferal blood mononuclear cells (PBMC) were prepared by centrifugation of blood from normal donors on a Ficoll-Hypaque 1077 (Sigma Chemical Co., St. Louis, MO) density gradient according to the manufacturer's recommendation. 10^5 PBMC (responders) were mixed with 10^5 irradiated (3000 rad) allogeneic PBMC (stimulators) and cultured in triplicate in round-bottom microtiter plates (200 µl/well). In some wells, cytotrophoblast conditioned medium (CM) from individual preparations of cytotrophoblast cells was added at a final concentration of 50% (v/v). Where specified, CM was preabsorbed with 20 to 0.15 µg/ml of a function-perturbing antibody to IL-10 (anti–IL-10, 19F1; Bejarano et al., 1992; Gastl et al., 1993) or an anti–β-Galactosidase antibody matched for IgG subclass (control IgG, GL117; Abrams et al., 1992) for 3 hours at 37°C prior to addition. On day 5, the medium was harvested and centrifuged for 10 min at 300 *g*. An ELISA specific for IFN-γ, developed in this laboratory (Abrams, 1995), was performed to determine levels of this cytokine in the culture medium. The sensitivity of the assay is 50 pg/ml. Data were analyzed using the Mann-Whitney U test for nonparametric distribution.

Figure 3-1. IL-10 levels secreted by cultured human cytotrophoblasts.

Cytotrophoblasts were isolated from pooled first or second trimester (TM) or individual term human placentas. 1 x 10^6 cells were cultured in 1 ml serum-free medium. After 24 h, conditioned medium was collected and levels of secreted IL-10 were quantified by ELISA. Each point represents the level of IL-10 in the conditioned medium of one preparation of cytotrophoblast cells. Diamonds signify means with 95% confidence intervals.

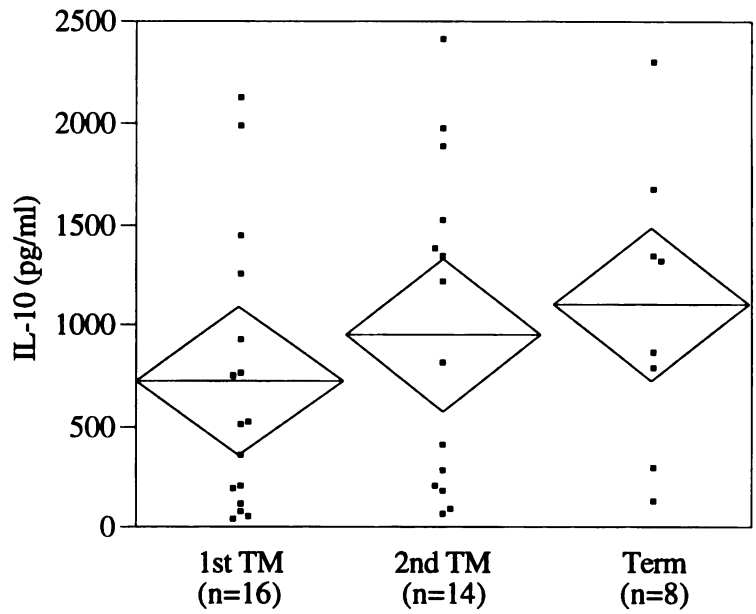

2500
2000
1500
1000
500
0
IL-10 (pg/ml)
1st TM
(n=16)
2nd TM
(n=14)
Term
(n=8)

Figure 3-1

Figure 3-2. Immunodetection of IL-10 in cultured human cytotrophoblasts.

2.5×10^5 first trimester cytotrophoblasts were cultured in 500 µl serum-free medium. After 12 h, cells were fixed and stained with mAbs to IL-10 (*a*) or IL-7 (*b*) as described in MATERIALS AND METHODS. Bar = 100 µm; inset, bar = 10 µm.

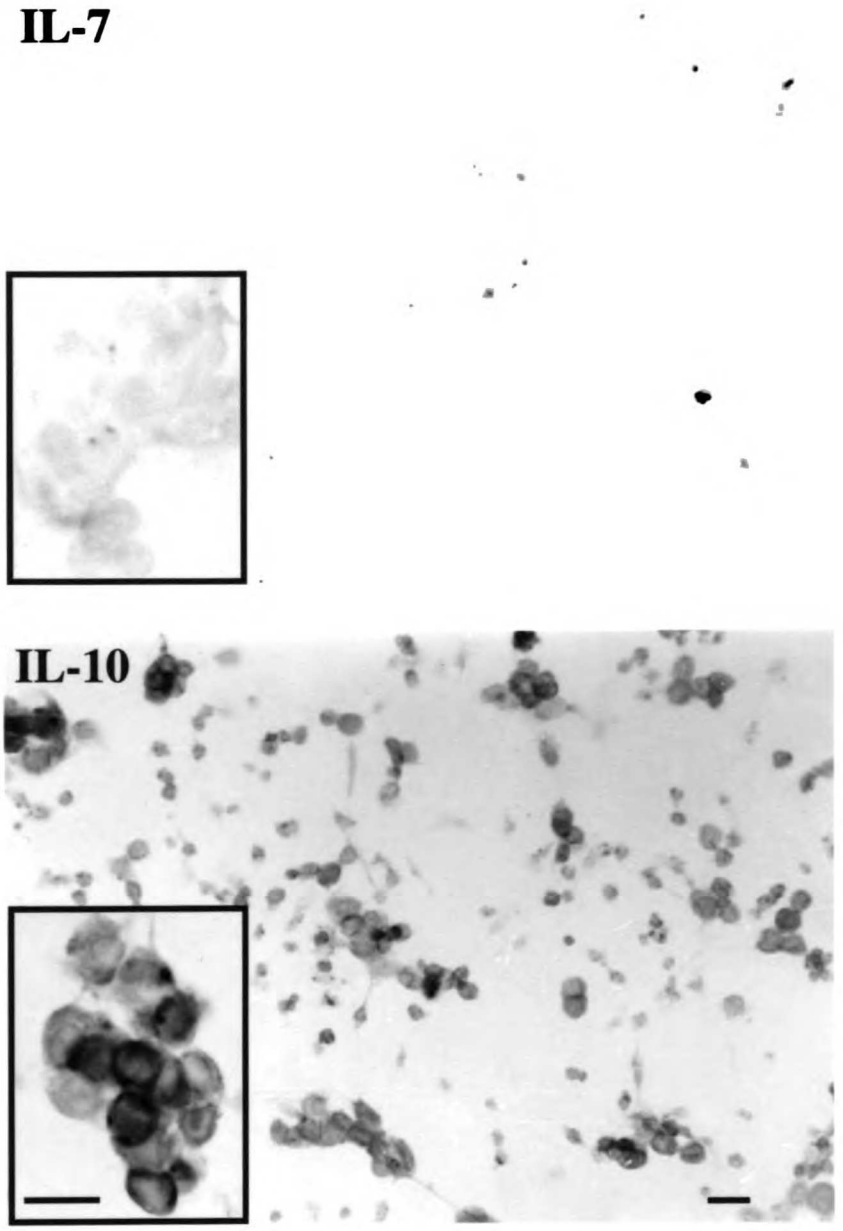


Figure 3-2

Figure 3-3. Human cytotrophoblasts synthesize IL-10 in vitro.

Term cytotrophoblasts were metabolically labeled for 12 h beginning at 0, 24, and 48 h in culture. Conditioned medium (CM) was collected and a cell extract (EX) was prepared. Samples were incubated overnight with mAbs to IL-10 or, as a negative control, a mAb to IL-7. The resulting immune complexes were separated on a 15% polyacrylamide gel and exposed to film for 3 days.

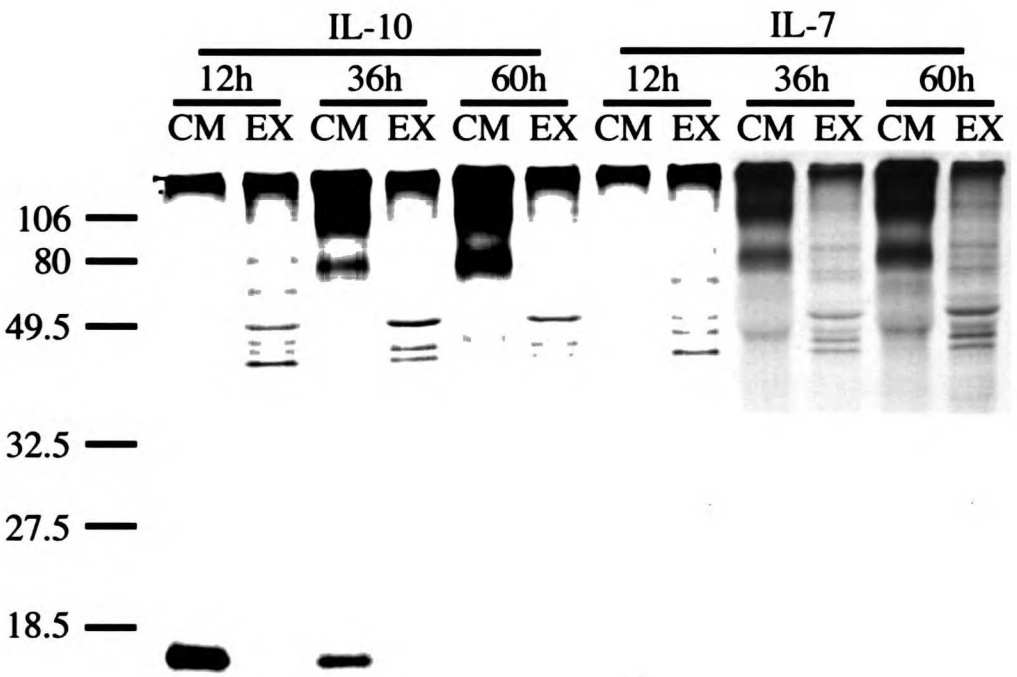

IL-10
IL-7
12h
36h
60h
12h
36h
60h
CM EX CM EX CM EX CM EX CM EX CM EX
106
80
49.5
32.5
27.5
18.5

Figure 3-3

Figure 3-4. Relative levels of IL-10 mRNA in human placental cytotrophoblasts over time in culture.

Placental cytotrophoblasts were isolated as described (MATERIALS AND METHODS) from pooled first or second trimester (TM) or term placentas and RNA was purified and reverse transcribed. cDNA was amplified by PCR in the presence of the competitor plasmid, DC10 (see MATERIALS AND METHODS), allowing quantification of the relative levels of IL-10 mRNA by comparison of the relative amounts of the larger competitor sequence (upper band in each lane) and the wild-type transcript (lower band in each lane). The equal relative intensities of cDNA and competitor HPRT amplimers in each sample (upper panel) indicate that an equal amount of input cDNA was added to each reaction. IL-10 transcripts were then quantitated by using these equalized amounts of input cDNA in PCR reactions containing a constant amount of DC10 (lower panel). As mRNA levels decrease, the larger competitor construct is preferentially amplified, resulting in a brighter band. Results are representative of two experiments.

Figure 3-4

Figure 3-5. IL-10 in cytotrophoblast conditioned medium inhibits IFN-γ secretion in a primary mixed lymphocyte reaction (MLR). Cytotrophoblast conditioned medium (CTB CM) was collected after 12 h from individual preparations of cells, and absolute amounts of IL-10 in each sample were determined by ELISA. Alloreactive lymphocytes were cultured in triplicate as described (MATERIALS AND METHODS) in the presence of RPMI alone (white bar) or 50% (v/v) CTB CM from individual cell preparations either untreated (black bars) or preabsorbed with 10 μg/ml of a neutralizing mAb to IL-10 (hatched bars). After 5 days, conditioned medium was collected and IFN-γ levels in triplicate cultures were determined by ELISA. Data of all experiments are presented as mean ± SEM. Asteriscs denote significant increases in IFN-γ secretion ($p < 0.05$ by the Mann-Whitney U test for nonparametric distribution).

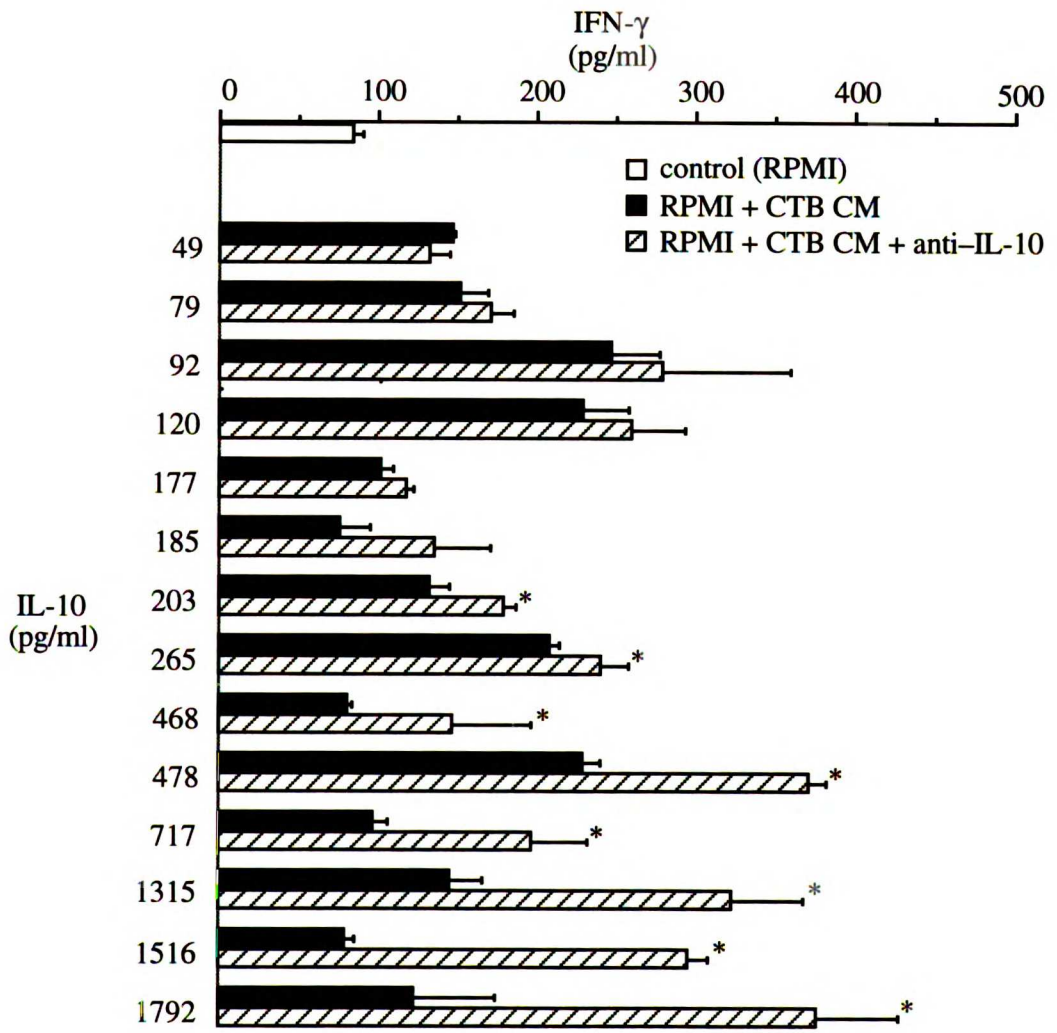

IFN-γ
(pg/ml)
0
100
200
300
400
500
control (RPMI)
RPMI + CTB CM
RPMI + CTB CM + anti–IL-10
IL-10
(pg/ml)
49
79
92
120
177
185
203
265
468
478
717
1315
1516
1792
*

Figure 3-5

Figure 3-6. Dose-response inhibition of IFN-γ secretion by cytotrophoblast IL-10. Conditioned medium (CM) was collected from cytotrophoblast cells after 12 h in culture. Alloreactive lymphocytes were cultured in triplicate as described (MATERIALS AND METHODS) in the presence of 50% (v/v) CM or 1000 pg/ml rIL-10, each preabsorbed with 20 to 0.15 μg/ml of a neutralizing mAb to IL-10 (anti–IL-10; A) or an isotype-matched control antibody (control IgG; B). Results shown are from two individual experiments and represent mean ± SEM.

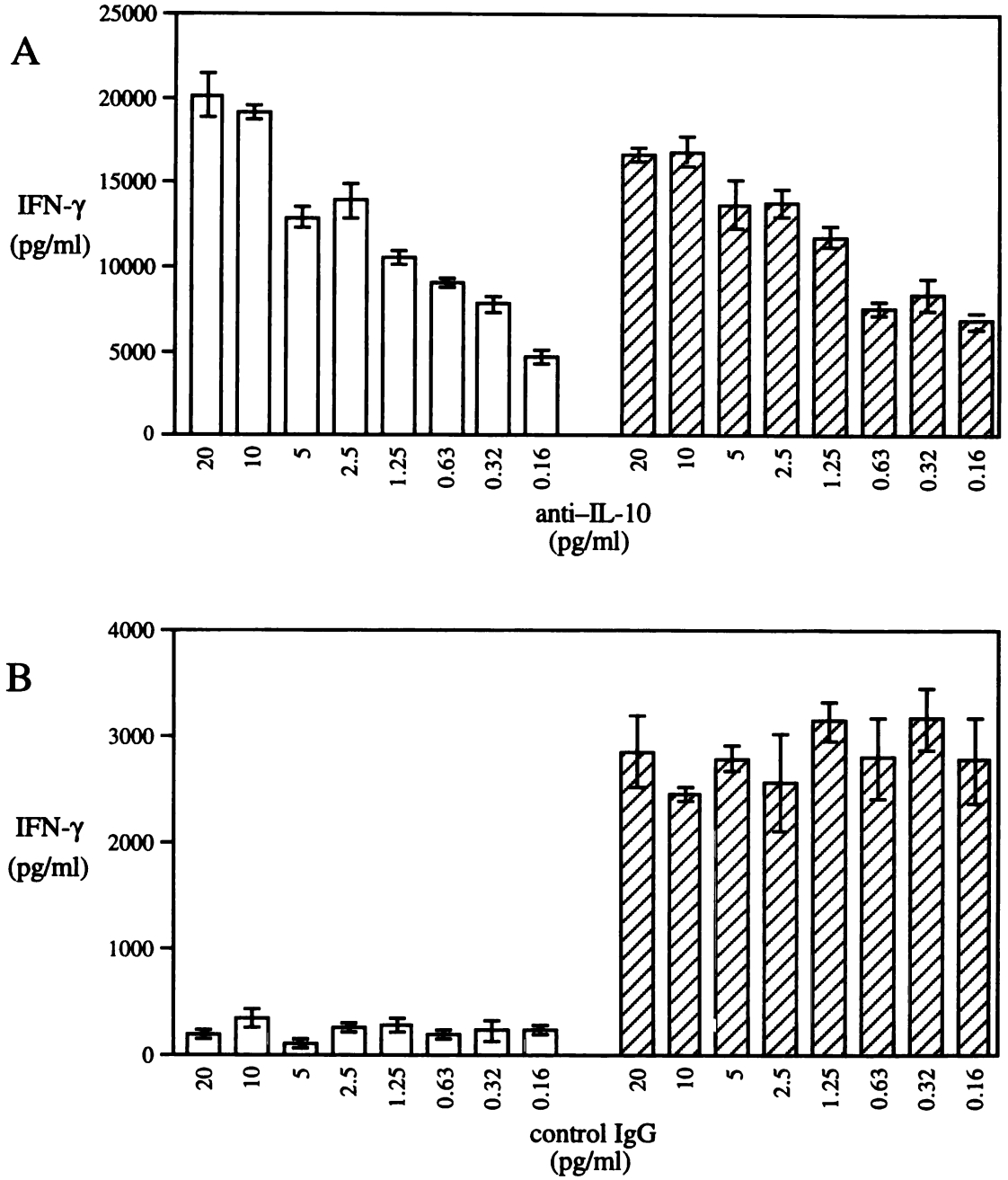

A
25000
20000
15000
10000
5000
0
IFN-γ
(pg/ml)
20
10
5
2.5
1.25
0.63
0.32
0.16
anti–IL-10
(pg/ml)
B
4000
3000
2000
1000
0
IFN-γ
(pg/ml)
control IgG
(pg/ml)
1000 pg/ml rIL-10
CTB CM

Figure 3-6

CHAPTER FOUR

IL-10 is an Autocrine Inhibitor of Human Placental Cytotrophoblast MMP-9 Production and Invasion

Abstract

During human placentation, fetal cytotrophoblast stem cells differentiate and then invade the uterine wall and its associated spiral arteries. This process anchors the placenta to the uterus and supplies maternal blood to the fetus. Cytotrophoblast invasion *in vitro* requires the expression of matrix metalloproteinase-9 (MMP-9). Recently, we showed that cytotrophoblasts produce interleukin-10 (IL-10), a potent immunomodulatory cytokine that could have paracrine effects on the maternal immune system [Roth et al. (1996) *J. Exp. Med.* 184, 539-548]. IL-10 synthesis is dramatically downregulated after the first 12 h of culture, while MMP-9 secretion is rapidly upregulated and the cells acquire an invasive phenotype. These observations prompted us to investigate whether IL-10 is an autocrine regulator of cytotrophoblast MMP-9 production. We found that the cells expressed IL-10 receptor mRNA, suggesting that autocrine effects are possible. Adding recombinant IL-10 to cytotrophoblast cultures significantly decreased the cells' MMP-9 expression at both protein and mRNA levels, but did not affect mRNA levels of the tissue inhibitor of metalloproteinase-3. Thus, IL-10 may alter the proteinase/inhibitor balance. IL-10 treatment further caused a net decrease in MMP activity, thereby reducing cytotrophoblast invasiveness. An antibody that neutralized endogenous IL-10 function had the opposite effect in all experiments. Together, these data suggest that IL-10 is an autocrine inhibitor of cytotrophoblast MMP-9 activity and invasiveness.

Introduction

Successful formation of the human placenta requires the correct differentiation of specialized fetal cells termed cytotrophoblasts (reviewed in Cross et al., 1994). Cytotrophoblast stem cells are attached to an extensive basement membrane that surrounds the stromal core of two types of chorionic villi. In floating villi, cytotrophoblasts differentiate by fusing to form an overlying layer of multinucleate syncytiotrophoblasts. These cells are in contact with maternal arterial blood that bathes the floating villi. The primary function of the syncytium is to perform nutrient, waste, and gas exchange between the maternal and fetal circulations. In anchoring villi, a subset of cytotrophoblasts at the distal tips of the villi differentiate by leaving their basement membrane and invading the uterus. These invasive cytotrophoblasts populate the decidualized endometrium and the first third of the myometrium, replacing the walls of uterine spiral arterioles in these regions (Brosens et al., 1972; Damsky et al., 1992). This unusual process anchors the placenta to the uterus and supplies the fetus with maternal blood. Cytotrophoblast invasion must be tightly regulated, because errors in the depth of uterine penetration can have severe negative effects on pregnancy outcome. Disorders such as preeclampsia (Zhou et al., 1993) and approximately half the cases of intrauterine growth retardation (Gerretsen et al., 1981) are associated with abnormally shallow cytotrophoblast invasion. Conversely, invasion that is overly extensive can result in both benign and malignant trophoblast tumors (Berkowitz and Goldstein, 1996).

Control of cytotrophoblast invasion requires the developmental regulation of several classes of molecules, including those involved in adhesion and proteolysis. The expression of cell-cell (*e.g.*, cadherin, Ig superfamily) and cell-extracellular matrix (ECM) adhesion molecules (*e.g.*, integrin receptors) is normally extensively modulated as cytotrophoblasts invade the uterine wall (Aplin, 1991; Damsky et al., 1992; Fisher et al., 1989). In

preeclampsia, where placentation is shallow, differentiating/invading cytotrophoblasts fail to correctly modulate members of all three adhesion molecule families (Lim et al., 1997; Zhou et al., 1993; Zhou et al., 1997). With regard to proteinases, human cytotrophoblasts produce both the urokinase-type plasminogen activator (Queenan et al., 1987) and matrix metalloproteinase-9 (MMP-9; Fisher et al., 1989). While urokinase-type plasminogen activator does not appear to directly mediate cytotrophoblast invasion *in vitro*, MMP-9 activity is absolutely required for cytotrophoblast degradation of and invasion through Matrigel (Librach et al., 1991). Like the adhesion molecules that are critical to invasion, MMP-9 expression is misregulated in preeclampsia (Lim et al., 1997).

MMPs are a family of ECM-degrading enzymes that share common structural domains (reviewed in Matrisian, 1990; Sang and Douglas, 1996). These proteinases are secreted as inactive zymogens and are activated by proteolytic cleavage. MMPs require Ca^{2+} binding for structural integrity and Zn^{2+} for catalysis. Consequently, their activity can be inhibited by chelating agents *in vitro* (Murphy and Docherty, 1992). *In vivo*, MMP induction in areas of matrix remodeling is under the tight control of several regulatory mechanisms. Proteinase abundance is regulated at the transcriptional level, as well as post-transcriptionally, by changes in mRNA stability. After secretion, MMP enzymatic activity is controlled by interactions with proteolytic activators (*e.g.*, membrane-type MMP) and inhibitors (*e.g.*, tissue inhibitors of metalloproteinases or TIMPs). This high degree of regulation may reflect the important roles MMPs play in normal tissue remodeling processes such as organogenesis and ovulation. The abundance and activity of family members is also increased in a variety of pathological processes such as inflammation and tumor metastasis (Ries and Petrides, 1995).

Cytokines and growth factors involved in the regulation of these normal and pathological events have been shown to modulate MMP expression and proteolytic activity (Birkedal-

Hansen, 1993; Ries and Petrides, 1995). MMP-9 production is upregulated by interleukin (IL)-1β in chondrocytes (Ogata et al., 1992), synovial fibroblasts (Unemori et al., 1991), and keratinocytes (Lyons et al., 1993). Transforming growth factor-ß also increases MMP-9 activity in keratinocytes (Salo et al., 1991), as well as in monocytes (Wahl et al., 1993). MMP-9 is also upregulated by tumor necrosis factor-α in both monocytes (Watanabe et al., 1993) and cervical fibroblasts (Sato et al., 1996), whereas keratinocyte MMP-9 is upregulated by epidermal growth factor and transforming growth factor-α (Lyons et al., 1993). The localized secretion of proinflammatory cytokines can thus lead to tissue destruction in disorders such as rheumatoid arthritis and periodontal disease (Ries and Petrides, 1995). Among the cytokines that act to downregulate proteinase activity, both IL-4 (Lacraz et al., 1992) and IL-10 (Lacraz et al., 1995; Mertz et al., 1994) have been shown to inhibit MMP-9 production in monocytes and macrophages. Thus, cytokines play an important role in the control of MMP-9 activity in a variety of cell types.

Several of these cytokines and growth factors are also found at the maternal-fetal interface, making them likely candidates for regulating cytotrophoblast MMP-9 expression and/or activity. We have previously reported that human cytotrophoblasts produce IL-1ß in culture, and that this cytokine is an autocrine stimulator of MMP-9 secretion and invasion (Librach et al., 1994). We hypothesized that the highly aggressive, yet precisely regulated nature of cytotrophoblast invasion was evidence that the cells had analogous mechanisms for inhibiting MMP activity. Of the cytokines known to downregulate MMP-9 in other cell types, we recently showed that IL-10 is spontaneously produced in substantial quantities by human cytotrophoblasts *in vitro* (Roth et al., 1996). IL-10 in cytotrophoblast conditioned medium can inhibit an allogeneic immune reaction, which suggests IL-10 may be important in protecting the fetus from a potentially deleterious maternal immune response *in vivo*. IL-10 production is significantly downregulated after 12 h in culture, at the time when cytotrophoblasts upregulate MMP-9 and differentiate into invasive cells.

Because the secretion patterns of IL-10 and MMP-9 are inversely related, we undertook the present study to determine whether IL-10 is involved in the control of cytotrophoblast MMP-9 production and, consequently, invasion.

Results

Human Cytotrophoblasts Express IL-10 Receptor mRNA. Our recent findings that cytotrophoblasts secrete substantial amounts of IL-10 *in vitro* and that production patterns of IL-10 and MMP-9 are inversely related (Roth et al., 1996) suggested that IL-10 may function as an autocrine regulator of cytotrophoblast MMP-9 expression and invasive activity. We began to test this hypothesis by using RT-PCR to determine whether cytotrophoblasts express the IL-10 receptor, and thus have the potential to respond to the IL-10 they produce. Amplification of HPRT cDNA verified RNA integrity in each of the samples (Figure 4-1). As positive and negative controls, cDNA for the IL-10 receptor was amplified from peripheral blood leukocytes and placental fibroblasts, respectively. After culturing for 12 h, cytotrophoblasts of all gestational ages expressed IL-10 receptor mRNA (Figure 4-1).

IL-10 Downregulates Cytotrophoblast MMP-9 Secretion in vitro. Next, we investigated whether maintaining cytotrophoblasts in high levels of IL-10 after endogenous cytokine secretion has ceased affects their MMP-9 secretion. In these experiments, the cells were plated in medium that contained 10 ng/ml rIL-10, and this medium was replaced every 24 h. Cytotrophoblasts were also incubated in medium that contained 10 μg/ml of either a function-perturbing anti–IL-10 mAb, or an irrelevant mAb (IgG) of the same isotype. Control cells were cultured in medium alone. The amount of MMP-9 in the conditioned medium of the variously treated cells was estimated at 24, 48 and 72 h by immunoblotting. Figure 4-2A shows the results of a typical experiment. The effects of cytokine and anti–IL-10 treatment became more pronounced over time. By 72 h, we consistently found that cytotrophoblasts cultured in rIL-10 secreted significantly less MMP-9 than cells incubated in medium alone. Conversely, cytotrophoblasts incubated in anti–IL-10 secreted significantly more MMP-9 than control cells, whereas adding an irrelevant IgG to the

medium had no effect. Figure 4-2B summarizes the results of five different experiments in which we analyzed, by immunoblotting, the effects of these various treatments on cytotrophoblast MMP-9 secretion. Compared with the controls (medium alone, irrelevant IgG), adding rIL-10 decreased cytotrophoblast MMP-9 secretion to 41 ± 10% of control levels ($P<0.05$), while anti-IL-10 treatment increased MMP-9 levels to 119 ± 7% percent ($P<0.05$).

We then determined, by immunoblotting, whether the cytokine and antibody effects we observed after 72 h of culture were dose-dependent. Figure 4-3 shows the results of a typical experiment. The addition of 1-100 ng/ml rIL-10 to cultured cytotrophoblasts resulted in a dose-dependent inhibition of MMP-9 secretion; the lowest dose had no effect and the highest dose had the greatest effect. Conversely, 1-100 μg/ml of anti-IL-10 resulted in a dose-dependent increase in MMP secretion. As expected, cells treated with 1-100 μg/ml of an irrelevant IgG secreted the same amount of MMP-9 as control cytotrophoblasts incubated in medium alone. Together, the data in figures 4-2 and 4-3 demonstrate that IL-10 is an autocrine inhibitor of cytotrophoblast MMP-9 production *in vitro*.

IL-10 Downregulates MMP-9 Production at a Pretranslational Level, but Does Not Affect TIMP-3 mRNA Expression. To better understand the regulatory mechanisms involved, we determined whether the addition of exogenous IL-10 to cytotrophoblasts affects their levels of MMP-9 mRNA. Three different preparations of cells were cultured in medium alone (control) or in medium containing either 10 ng/ml rIL-10, 10 μg/ml anti-IL-10 mAb, or 10 μg/ml of an irrelevant mAb (IgG). Medium containing these additives was replaced daily, and total RNA was extracted after 36 and 72 h. The RNA samples from each cell preparation were pooled, and MMP-9 mRNA levels were assessed by Northern blot hybridization (Figure 4-4). In accord with the regulation we observed at the protein level,

rIL-10 treatment decreased MMP-9 mRNA levels, whereas anti–IL-10 treatment increased them, and the observed effects became more pronounced over time. Normalized for RNA loading differences, densitometric analysis showed that exposing cytotrophoblasts to rIL-10 for 36 and 72 h caused a 15% and a 50% decrease in MMP-9 mRNA levels, respectively. Conversely, exposing cytotrophoblasts to anti–IL-10 for 36 and 72 h caused a 16% and a 46% increase in MMP-9 mRNA levels, respectively. MMP-9 mRNA levels in cells treated for the same length of time with an irrelevant IgG were no different from those of control cells (Figure 4-4). These results suggest that IL-10 can regulate cytotrophoblast MMP-9 production at the pretranslational level.

We were also interested in whether IL-10 has similar effects on MMP-9 mRNA in other cell types. We chose to study U937 cells, a line of human mononuclear phagocytes that produce MMP-9 (Morodomi et al., 1992) and express the IL-10 receptor (Takeshita et al., 1996). U937 cells were cultured in medium alone (control) or in medium containing 10 ng/ml rIL-10, 10 μg/ml anti-IL-10 mAb, or 10 μg/ml of an irrelevant IgG for 72 h. Northern blot hybridization showed that neither IL-10 nor antibody treatment significantly affected MMP-9 mRNA levels (data not shown). This was in contrast to previous studies that show the same amount of IL-10 downregulates MMP-9 production by primary monocytes and macrophages (Lacraz et al., 1995). Taken together, our results and those of other investigators suggest that the effects of IL-10 on MMP-9 production are cell-type-specific.

Next, we determined whether IL-10 regulates cytotrophoblast TIMP production. These cells make very little TIMP-1 and -2. However, expression of TIMP-3 protein and mRNA, like that of MMP-9, is dramatically upregulated in the first 12 h in culture (K. Bass *et al.*, in preparation). Since TIMP-3 can inhibit MMP-9 activity (Apte et al., 1995), changes in its levels could affect cytotrophoblast proteolytic capacity. To determine

whether IL-10 regulates cytotrophoblast TIMP-3 mRNA expression, the filter shown in figure 4-4 was stripped and hybridized with a TIMP-3–specific probe. When normalized for differences in RNA loading, densitometric analysis showed that neither IL-10 nor anti-IL-10 treatment for 72 h changed TIMP-3 mRNA levels. These data suggest that IL-10 changes the MMP/TIMP balance in favor of the inhibitor. As a consequence, net cytotrophoblast MMP activity should decrease.

IL-10 Downregulates Cytotrophoblast MMP-9 Activity. We used two methods to assess the effects of IL-10 on cytotrophoblast MMP activity, beginning with zymography for an initial estimate of changes. Consistent with our previous observations (Fisher et al., 1989; Librach et al., 1991), the major gelatinolytic activity was in the 92-kDa region, and none of the cytokine or antibody treatments induced other activities (data not shown). The effects of rIL-10 and anti–IL-10 on cytotrophoblast MMP-9 activity paralleled their effects on MMP-9 protein expression, as shown by immunoblotting (Figure 4-2A). Again, these changes were somewhat variable during the first two days of culture (data not shown), but at 72 h we found consistent differences. Figure 4-5A shows the results of a typical experiment. Cytotrophoblasts cultured in rIL-10 secreted considerably less 92-kDa gelatinolytic activity than control cells, and cytotrophoblasts incubated in anti-IL-10 secreted considerably more 92-kDa gelatinolytic activity. Figure 4-5B summarizes the results of five experiments. After cytokine treatment for 72 h, MMP-9 activity declined to $62 \pm 9\%$ of control levels ($P<0.05$), while anti–IL-10 treatment caused MMP-9 activity to increase by $48 \pm 12\%$ ($P<0.05$). Addition of the control, isotype-matched IgG did not affect gelatin degradation. Finally, these effects on cytotrophoblast MMP-9 activity depended on the dose of IL-10 or anti–IL-10 that was added to the cells. Figure 4-6 shows results typical of the three experiments we did. The addition of 1-100 ng/ml rIL-10 to cultured cytotrophoblasts resulted in a dose-dependent inhibition of MMP-9 gelatinolytic activity; the lowest dose had no effect and the higher doses had the greatest effects.

Conversely, 1-100 μg/ml of anti-IL-10 resulted in a dose-dependent increase in MMP gelatinolytic activity. Again, the control IgG had no effect.

Because zymography is not rigorously quantitative, we also used a very sensitive chromogenic assay to quantify the effects of IL-10 and anti-IL-10 on cytotrophoblast MMP activity (Weingarten et al., 1985). Figure 4-7 shows the results of five such experiments. After 72 h in culture, control conditioned medium and medium that contained an irrelevant IgG contained the same levels of MMP activity. As predicted from the zymogram data, rIL-10 treatment resulted in a 33 ± 9.7% decrease in MMP activity from control levels ($P<0.05$). Conversely, anti–IL-10 stimulated MMP activity by 43 ± 15% ($P<0.05$). Together, the results of the zymogram and chromogenic assays suggest that IL-10 is a negative regulator of cytotrophoblast MMP-9 activity.

IL-10 Inibits Cytotrophoblast Invasive Capacity in vitro. Our lab has shown that cytotrophoblast MMP-9 activity is required for one of the cells' most important and unusual functions, namely invasion (Librach et al., 1991). To test the functional relevance of IL-10 regulation of MMP-9 expression and activity, we quantified the invasive capacity of cytotrophoblasts treated with cytokine, anti–IL-10, or control IgG *in vitro*. Specifically, we assayed the cells' ability to invade Matrigel-coated, porous polycarbonate filters. Initially, the cells send long processes through the filter pores (48-72 h). Soon thereafter, entire cell bodies emerge on the filter underside (72 h onward).

Figure 4-8A summarizes the results of three experiments in which we assayed the effects of IL-10 on the appearance of cytotrophoblast cell processes on the bottom of the filter. When the cells were cultured for 60 h in the presence of rIL-10, 64 ± 8% fewer cytotrophoblasts traversed the filter pores compared with cells cultured in medium alone ($P<0.05$). Conversely, neutralizing IL-10 function caused a 33 ± 7% increase in the number of cell

processes that emerged on the filter underside (anti–IL-10), while the irrelevant mAb (IgG) had no effect ($P<0.05$). Later in the culture period, we found that these same treatments also affected the number of cell bodies that emerged on the bottom of the filters. After 72 h, cytokeratin staining of control cultures and those treated with irrelevant IgG showed that most of the pores contained cell processes (Figure 4-8B). A few cell bodies were also visible on the bottom of the filter. In the presence of rIL-10, many fewer processes reached the pores and no cell bodies were visible. With anti-IL-10 treatment, invasion was enhanced such that many cell bodies were present on the filter underside. Together, these data show that IL-10 is an autocrine inhibitor of cytotrophoblast invasion *in vitro*.

Discussion

IL-10 is a pleiotropic cytokine whose known functions include the regulation of both immune responses and proteinase production. Among its potent immunosuppressive effects are the downregulation of proinflammatory cytokine synthesis and the inhibition of alloreactive T-cell proliferation (Moore et al., 1993). IL-10 has also been shown to regulate the expression of both serine proteinases (Ghildyal et al., 1992; Ghildyal et al., 1992) and matrix metalloproteinases (Lacraz et al., 1995; Mertz et al., 1994; Reitamo et al., 1994) in a variety of cell types. Proteinase activity is required for cytotrophoblast invasion of the uterine wall during placentation. At the same time, these allogeneic fetal cells must avoid maternal immune rejection. This suggested to us that cytotrophoblast IL-10 could play a role in the regulation of both of these biological processes. We previously described cytotrophoblast IL-10 synthesis *in vitro* and showed that this cytokine, present in the cells' conditioned medium, can suppress allogeneic T-lymphocyte reactivity (Roth et al., 1996). We now demonstrate that IL-10 acts via an autoregulatory mechanism to specifically inhibit cytotrophoblast MMP-9 production at the pretranslational level. The subsequent downregulation of proteinase secretion results in a decrease in MMP-9 activity; as a consequence, cytotrophoblast invasion is significantly compromised.

IL-10 thus represents an important link between placental immune protection of the fetus and cytotrophoblast invasion of the uterus. We find that cytotrophoblast stem cells produce IL-10 *in vivo* (I. Roth and D. Figueroa, unpublished observations). Our data show that IL-10 is downregulated as these cells differentiate, thus permitting the production of MMP-9 necessary for uterine invasion. In the absence of IL-10 secretion, the expression of HLA-G by invasive cytotrophoblasts in the uterine wall may protect these cells from maternal immune attack (Kovats et al., 1990; McMaster et al., 1995; Pazmany et al., 1996). However, if invading cytotrophoblasts encounter an immunologically hostile

environment, IL-10 production could be upregulated to serve two important functions: high cytokine levels will suppress a harmful immune reaction, and invasion will be restricted to minimize the number of cytotrophoblasts in contact with maternal leukocytes. In support of this hypothesis, preliminary observations suggest that IL-10 levels are markedly elevated in severe preeclampsia *in vivo* (I. Roth and S. Fisher, unpublished observations). This disorder is thought to have an immunological basis, and is characterized by abnormally shallow cytotrophoblast invasion of the uterine wall (Friedman et al., 1991).

Here we demonstrate that cytotrophoblast IL-10 acts via an autocrine mechanism to downregulate MMP-9 secretion without affecting TIMP-3 mRNA expression. Cytokine treatment thus increases the effective ratio of inhibitor-to-proteinase levels, resulting in a net decrease in proteolytic activity and invasion. In doing so, the consequences of suppressing proteinase production alone are amplified. These observations suggest that cytotrophoblast expression of MMP and TIMP mRNAs is regulated by disparate mechanisms. A similar phenomenon has previously been described in monocytes and macrophages. In these cells, treatment with interferon-γ (Shapiro et al., 1990), IL-4 (Lacraz et al., 1992), or IL-10 (Lacraz et al., 1995) has different effects on expression of MMP and TIMP mRNA. For example, IL-10 downregulates monocyte/macrophage MMP-9 mRNA but stimulates TIMP-1 expression (Lacraz et al., 1995). Together, these findings highlight the complex, cell-type-specific nature of cytokine regulation of ECM degradation.

Finally, cytotrophoblast invasion of the uterine wall is one of the critical first steps for successful human pregnancy. Because of its importance, this process is undoubtedly influenced by many redundant regulatory mechanisms. We have identified several molecular targets for both the spatial and temporal control of cytotrophoblast invasion (Cross et al., 1994; Damsky et al., 1993). In addition to MMP-9, cytotrophoblast expression of adhesion molecules, including integrins, cadherins, and Ig superfamily

members, is modulated in normal pregnancy (Damsky et al., 1993; Zhou et al., 1997). We have also shown that adhesion molecule expression is disregulated in preeclampsia, in which cytotrophoblast invasion is shallow (Zhou et al., 1993; Zhou et al., 1997) Thus, the interesting possibility exists that IL-10 also affects the production of these important molecules.

Materials and Methods

Cell Isolation and Culture. Cytotrophoblasts were isolated by published methods from first, second (Fisher et al., 1989; Librach et al., 1991), and third trimester (Kliman et al., 1986) human placentas. In all cases, contaminating leukocytes were removed by using an antibody to CD-45, a protein tyrosine phosphatase found on bone marrow-derived cells (Charbonneau et al., 1988), coupled to magnetic beads. Cytotrophoblast populations prepared in this way are 95-99% free of non-trophoblast cells as shown by the absence of classical HLA class I antigens (Kovats et al., 1990). Control cytotrophoblasts (1×10^6) were cultured in 1 ml serum-free medium (DME H-21 containing 2% Nutridoma [Boehringer Mannheim]) in 16-mm culture wells precoated with a basement membrane substrate (Matrigel; Collaborative Research Inc., Bedford, MA) diluted two parts to one part serum-free medium as described (Librach et al., 1991). Where specified, 1-100 ng/ml of recombinant IL-10 (rIL-10; R&D Systems), 1-100 μg/ml of a rat neutralizing monoclonal antibody (mAb) (IgG_{2a}) to IL-10 (anti–IL-10; 19F1 [Bejarano et al., 1992; Gastl et al., 1993]), or 1-100 μg/ml of a rat IgG_{2a} isotype control (IgG; GL117, anti-*E. coli* ß-galactosidase) were added to the cytotrophoblast culture medium. Medium containing these additives was replaced every 24 h.

Placental fibroblasts were isolated from first trimester placentas and cultured as previously described (Fisher et al., 1989). Peripheral blood leukocytes were prepared by centrifugation of blood from normal donors on a Ficoll-Hypaque 1077 (Sigma) density gradient according to the manufacturer's recommendation. The leukocytes were cultured in RPMI 1640 supplemented with 10% (v/v) heat-inactivated human AB serum at a density of 1×10^6 cells/ml culture medium.

U937 cells were maintained in RPMI-1640 supplemented with 10% (v/v) fetal calf serum. To induce differentiation and MMP-9 secretion, control cells were cultured at a concentration of 1 x 10^6/ml in RPMI containing 50 ng/ml phorbol 12-myristate 13-acetate (Sigma) and 0.2% lactalbumin hydrolysate (Gibco BRL). As described in the Results section, 10 ng/ml rIL-10, 10 µg/ml 19F1, or 10 µg/ml control IgG were added to the cultures, and medium containing these additives was replaced daily.

Reverse transcriptase-polymerase chain reaction (RT-PCR) for Detection of IL-10 Receptor mRNA. Total RNA was isolated from 10^6 cells by using RNAzol B™ (Biotecx Laboratories, Inc., Houston, TX) according to the manufacturer's recommendations. RNA was reverse transcribed by using murine Moloney leukemia virus reverse transcriptase (Gibco BRL) and random hexamer primers (Sigma) as previously described (Reiner et al., 1993). PCR was performed using primers for either the constitutively expressed housekeeping gene, hypoxanthine-guanine phosphoribosyltransferase (HPRT), as a control for RNA integrity, or the IL-10 receptor. The following primer sets were used: HPRT 3': CCTGCTGGATTACATCAAAGCACTG; HPRT 5': TCCAACACTTCGTGGGGTCCT; IL-10 receptor 3': AGAGACCCAAGACACCATCCACCCG; IL-10 receptor 5': AAAACTTCAGCCTCCTAACCTCTGG. Amplification products were visualized by electrophoresis on 3% ethidium bromide-stained agarose gels and photographed.

Immunoblotting. Cytotrophoblasts were cultured for 72 h as described. Conditioned medium (CM) was collected and centrifuged at 300 x *g* for 10 min, then stored at –80°C until analysis by either immunoblotting, zymography, or a soluble assay of metalloproteinase activity (described below). For immunoblot analysis, CM (75% v/v) was solubilized in loading buffer, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred by blotting to nitrocellulose as described

(Librach et al., 1991). Nonspecific reactivity was blocked by incubating the blots for 1 h at 25°C in phosphate buffered saline (PBS) containing 0.05% Tween-20 (T-PBS) and 5% nonfat dried milk (Carnation). The nitrocellulose blots were then incubated for 2 h at 25°C with 5 μg/ml anti-MMP-9 antibody (7-11C; Oncogene Research Products, Cambridge, MA) suspended in T-PBS containing 5% nonfat dry milk. The blots were washed 6 times (10 min each time) in T-PBS, then incubated for 1 h at 25°C with horseradish peroxidase-conjugated rabbit anti-mouse IgG (Jackson Immunoresearch) diluted 1:3000 in T-PBS containing 5% nonfat dry milk. Blots were then washed six times (10 min each time) in T-PBS and rinsed once in PBS. An enhanced chemiluminescence system (Amersham Corp.) was used to detect antigen-antibody complexes, and the blot was exposed to film.

Northern Hybridization. Total RNA (10 μg) was separated by formaldehyde-agarose gel electrophoresis and transferred to Nytran membranes (Schleicher and Schuell, Inc., Keene, NH). In all experiments, gels were stained with acridine orange before transfer to ensure integrity of the RNA samples, and to confirm that equal amounts of RNA had been loaded onto each lane. A TIMP-3–specific cDNA probe was synthesized by random priming of a 515-bp fragment of the cDNA (nucleotides 369-884 [Silbiger et al., 1994]). An MMP-9–specific probe was synthesized by PCR amplification of U937 cell cDNA to generate a 382-bp fragment (nucleotides 15-397 [Chien et al., 1996]). Nytran membranes were analyzed by Northern blot hybridization as previously described (McMaster et al., 1995).

Substrate Gel Zymography. Cytotrophoblast or U937 cell CM (75% v/v) was solubilized in nonreducing sample buffer and separated by SDS-PAGE using 10% acrylamide gels containing 5 mg/ml gelatin (Sigma). After electrophoresis, proteins were renatured by incubating the gel in 50 mM Tris-HCl, pH 7.6, containing 2.5% Triton X-100 for 1 h at 25°C to remove SDS. Metalloproteinases were then activated by incubating the gel overnight at 37°C in 50 mM Tris-HCl, pH 7.8, containing 150 mM NaCl and 5 mM

$CaCl_2$. To visualize proteinase activity, the gel was stained with Coomassie blue. Clear bands corresponded to areas where gelatin digestion took place. The gels were dried onto filter supports and photographed .

Soluble Assay of Metalloproteinase Activity. Metalloproteinase activity in cytotrophoblast CM was measured using the thiopeptolide substrate Ac-Pro-Leu-Gly-$SCH[CH_2CH(CH_3)_2]CO$-Leu-Gly-OC_2H_5 (Bachem, Philadelphia, PA) in a spectrophotometric assay (Weingarten et al., 1985). CM (25%, v/v) was added to a reaction mixture containing 40 µM thiopeptolide and 800 µM of the chromophore 4,4'-dithiodipyridine (Sigma) in 50 mM HEPES buffer, pH 7.0, with 10 mM $CaCl_2$. The reaction mixture was incubated at 25°C, and absorbance (A_{324}) was read at intervals of 5 min for 30 min. The rate of substrate degradation is a measure of total MMP activity. To verify that the activity measured reflected that of metalloproteinases, other CM samples were incubated with 1 mM 1,10-phenanthroline (Sigma) for 10 min at 25°C prior to addition of substrate. In all cases, this abolished the observed activity.

Invasion Assay. To quantitate cytotrophoblast invasion, Transwell polycarbonate filter inserts (6.5 mm; Costar) with 8 µm pores were coated with 10 µl Matrigel, and second trimester cells (2.5×10^5 cells/250 µl serum-free medium) were plated on top of each filter. Where specified, 10 ng/ml rIL-10, 10 µg/ml anti–IL-10, or 10 µg/ml of control IgG was added to the culture medium both above and below filter inserts and replaced every 24 h. At 60 or 72 h, filters were rinsed with PBS and the cells were fixed in 3% paraformaldehyde for 1 h at 25°C. After three 10-min washes in PBS, cell membranes were permeabilized with methanol for 5 min at –20°C. The filters were washed in PBS and then incubated in PBS containing 1% bovine serum albumin (PBS-BSA) for 1 h at 25°C to block nonspecific antibody reactivity. Cytotrophoblasts were exposed to an antibody to cytokeratin (1:100 v/v in PBS-BSA; 7D3 (Fisher et al., 1989)) for 2 h at 25°C, then

washed thoroughly with PBS. To detect cytotrophoblast cell bodies and processes, the filters were incubated with a rhodamine-conjugated anti-rat IgG (1:2000 v/v in PBS-BSA; Jackson Immunoresearch) for 1 h at 25°C, then washed again in PBS. Each of triplicate filters was cut from the Transwell inserts with a scalpel and mounted on a slide with the underside of the filter facing up. Cell bodies and processes that had migrated through the filter pores and had reached the underside were visualized by fluorescence microscopy and counted, then photographed.

Statistical Analyses. Thetatistical significance of the data was analyzed using Student's paired *t* test. The significance level was set at $P<0.05$ for differences from control.

Figure 4-1: *Human cytotrophoblasts express IL-10 receptor mRNA in vitro.* RNA was extracted from peripheral blood leukocytes (PBL), first and second trimester (TM) and term placental cytotrophoblasts, and first trimester placental fibroblasts (PFib) and reverse transcribed. The cDNA for HPRT or the IL-10 receptor (IL-10R) was amplified by PCR. The products were separated by electrophoresis in agarose gels, visualized by ethidium bromide staining, and photographed. RNA integrity was assured by the amplification of HPRT in all samples.

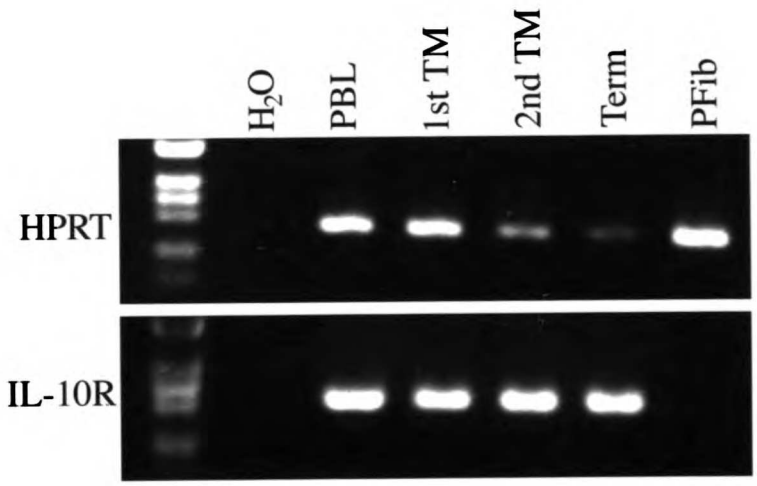


Figure 4-1

Figure 4-2: *Cytotrophoblast IL-10 is a negative regulator of MMP-9 secretion.* Second trimester cytotrophoblasts were cultured for the indicated times in culture medium alone (C), 10 ng/ml recombinant IL-10 (rIL-10), 10 μg/ml of a function-perturbing mAb to IL-10 (anti–IL-10), or 10 μg/ml of an isotype-matched irrelevant antibody (IgG). (*A*) Conditioned media (CM) were analyzed by immunoblotting using an antibody specific for MMP-9. (*B*) The relative intensities of MMP-9 bands after 72 h from five experiments were measured by densitometric analysis. Data are expressed as percent of control. Values represent mean ± SEM ; *$P < 0.05$ compared with control by paired *t* test.

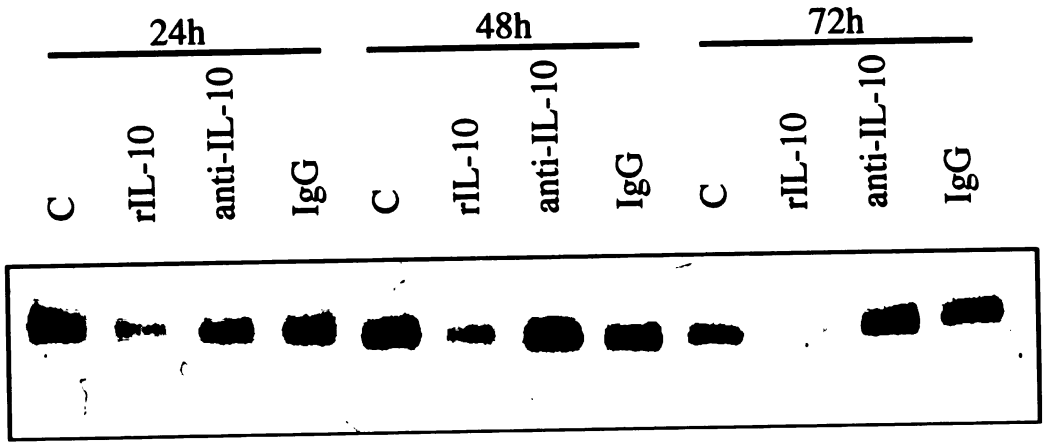

24h
48h
72h
C
rIL-10
anti-IL-10
IgG
C
rIL-10
anti-IL-10
IgG
C
rIL-10
anti-IL-10
IgG

Figure 4-2A

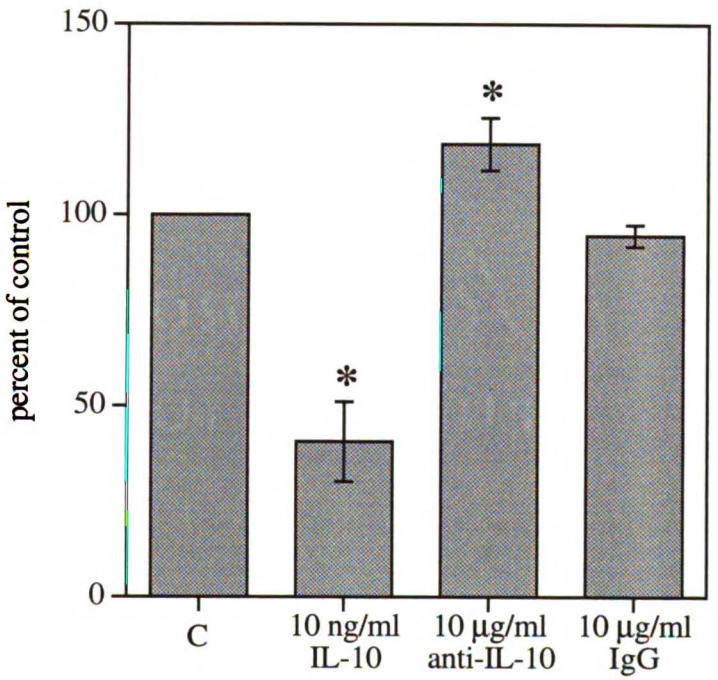

150
100
50
0
percent of control
*
*
C
10 ng/ml IL-10
10 µg/ml anti-IL-10
10 µg/ml IgG

Figure 4-2B

Figure 4-3: *The effect of IL-10 on MMP-9 secretion is dose dependent.* Second trimester cytotrophoblasts were cultured in culture medium alone (C) or with decreasing concentrations of rIL-10 (100–1 ng/ml), anti–IL-10 (100–1 μg/ml), or IgG (100–1 μg/ml). CM were collected after 72 h in culture and analyzed by immunoblotting.

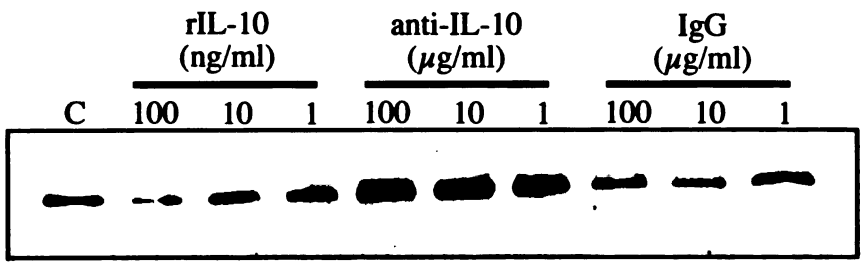


Figure 4-3

Figure 4-4: *IL-10 downregulates cytotrophoblast MMP-9 mRNA expression, but does not affect TIMP-3 mRNA levels.* Second trimester cytotrophoblasts were cultured in culture medium alone (C), 10 ng/ml rIL-10, 10 μg/ml anti–IL-10, or 10 μg/ml IgG. Total RNA was extracted from cytotrophoblasts after 36 and 72 h in culture. RNA from three different preparations of cells was pooled and subjected to Northern hybridization using a cDNA probe for MMP-9. This blot was then stripped and probed for TIMP-3 as described in Experimental Procedures.

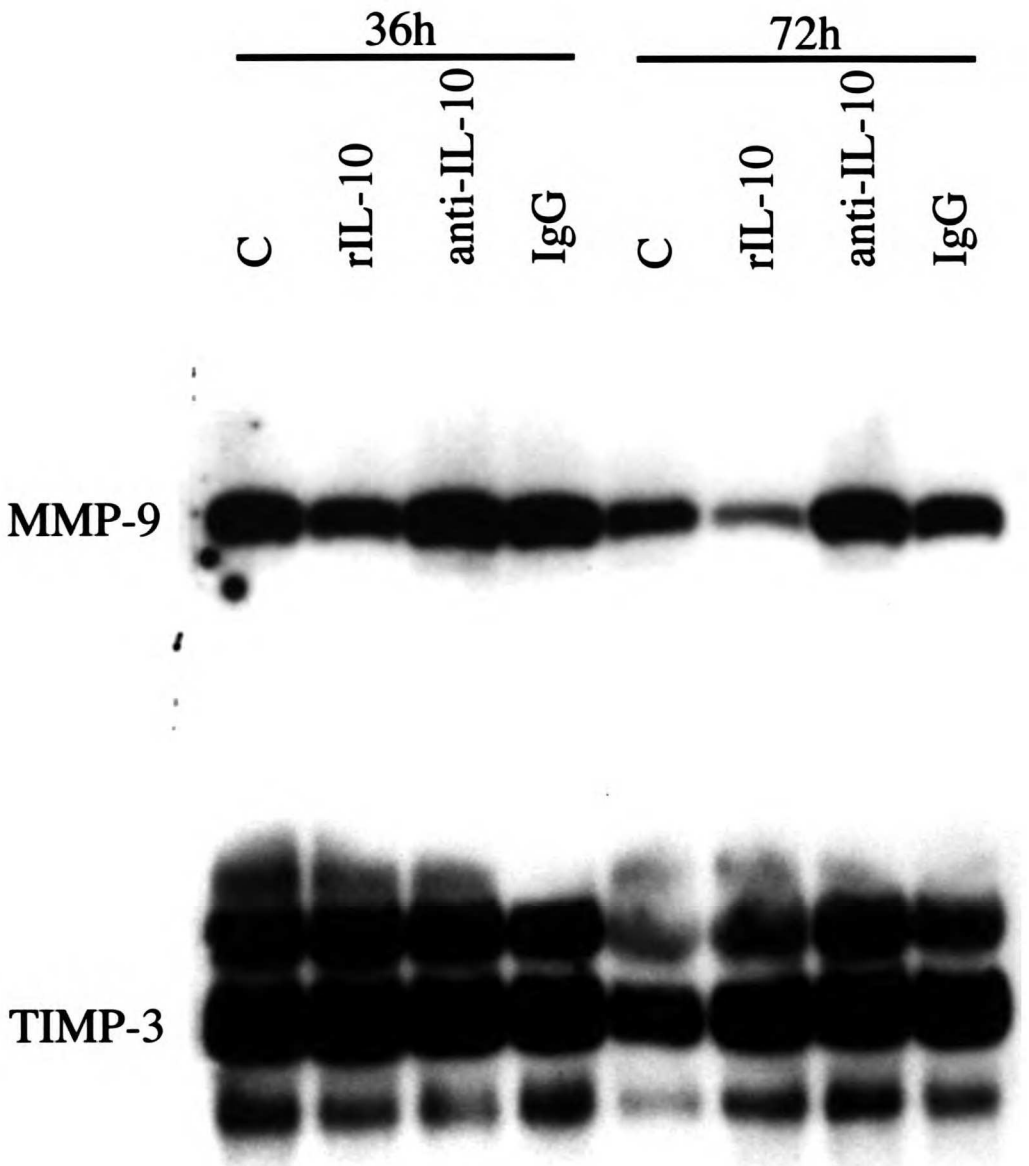


Figure 4-4

Figure 4-5: *Cytotrophoblast IL-10 is a negative regulator of MMP-9 activity.* Second trimester cytotrophoblasts were cultured in culture medium alone (C), 10 ng/ml rIL-10, 10 μg/ml anti–IL-10, or 10 μg/ml IgG. CM was collected after 72 h and analyzed by zymography as described in Experimental Procedures. (*A*) Bands show areas of gelatinolysis. (*B*) The relative intensities of bands from five experiments were measured by densitometric analysis. Data are expressed as percent of control. Values represent mean ± SEM ; *P < 0.05 compared with control by paired *t* test.

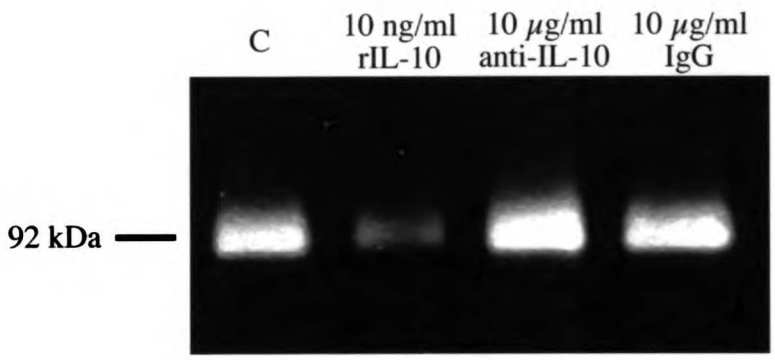


Figure 4-5A

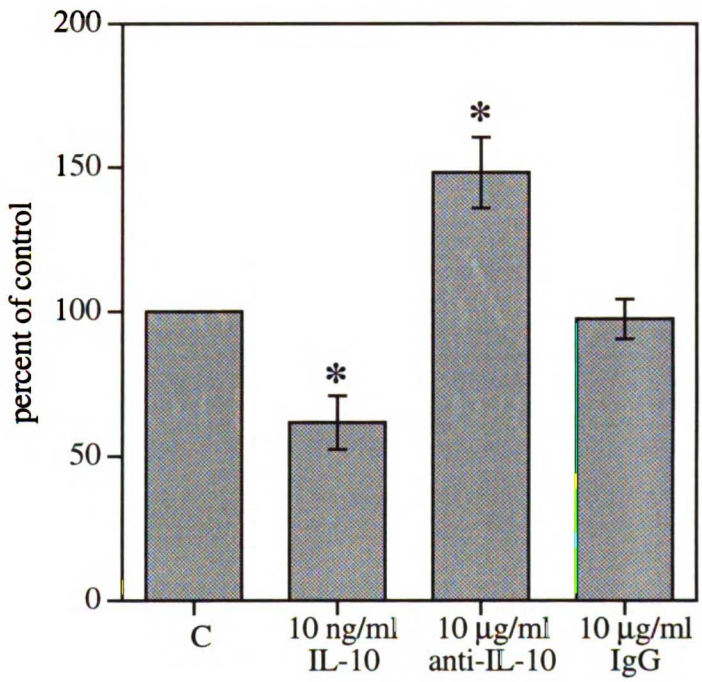

200
150
100
50
0
percent of control
*
*
C
10 ng/ml IL-10
10 µg/ml anti-IL-10
10 µg/ml IgG

Figure 4-5B

Figure 4-6: *The effect of IL-10 on MMP-9 activity is dose-dependent.* Second trimester cytotrophoblasts were cultured in culture medium alone (C) or with decreasing concentrations of rIL-10 (100–1 ng/ml), anti–IL-10 (100–1 μg/ml), or IgG (100–1 μg/ml). CM was collected after 72 h and analyzed by zymography (see Experimental Procedures). Bands show areas of gelatinolysis.

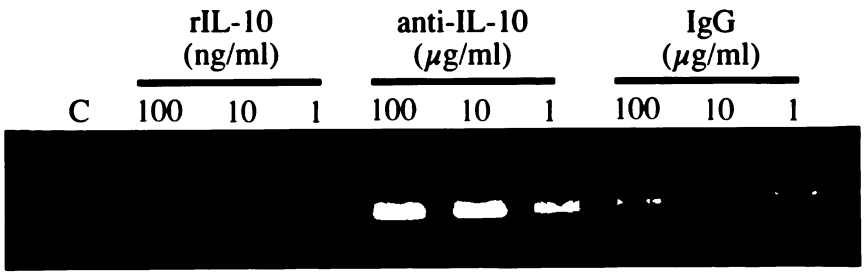


Figure 4-6

Figure 4-7: *IL-10 downregulates cytotrophoblast MMP-9 activity.* Second trimester cytotrophoblasts were cultured in culture medium alone (C) or medium containing 10 ng/ml rIL-10, 10 μg/ml anti–IL-10, or 10 μg/ml IgG. CM was collected after 72 h and added (25% v/v) to a synthetic thiopeptolide MMP substrate as described in Experimental Procedures. Hydrolysis of this substrate was measured over 30 min. Data are expressed as percent of control. Values represent mean ± SEM of five experiments; *$P < 0.05$ compared with control by paired *t* test.

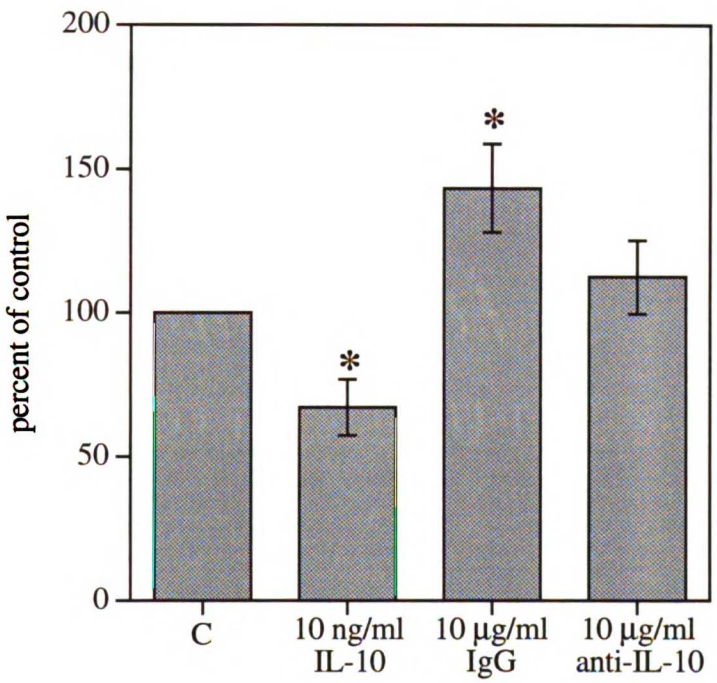

200
150
100
50
0
percent of control
*
*
C
10 ng/ml IL-10
10 µg/ml IgG
10 µg/ml anti-IL-10

Figure 4-7

Figure 4-8: *IL-10 inhibits cytotrophoblast invasion in vitro.* Second trimester cytotrophoblasts were cultured on top of Matrigel-coated porous polycarbonate filter supports in culture medium alone (C) or medium containing 10 ng/ml rIL-10, 10 μg/ml anti–IL-10, or 10 μg/ml IgG (see Experimental Procedures). (*A*) Some cultures were fixed after 60 h and stained with cytokeratin antibodies and cytotrophoblasts on the filter underside were counted. Data are expressed as percent of control. Values represent mean ± SEM from three experiments; *$P < 0.05$ compared with control by paired *t* test. (*B*) In other cultures, cytotrophoblasts were fixed after 72 h, then subjected to immunohistochemistry and photographed. The photomicrograph shown in *B* is typical of the results of the three experiments. Bar = 15 μm.

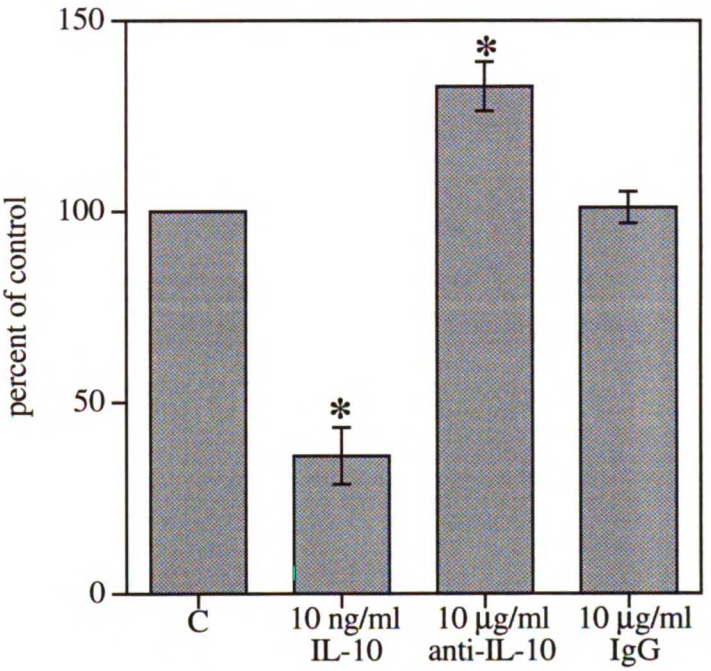

150
100
50
0
percent of control
*
*
C
10 ng/ml IL-10
10 µg/ml anti-IL-10
10 µg/ml IgG

Figure 4-8A

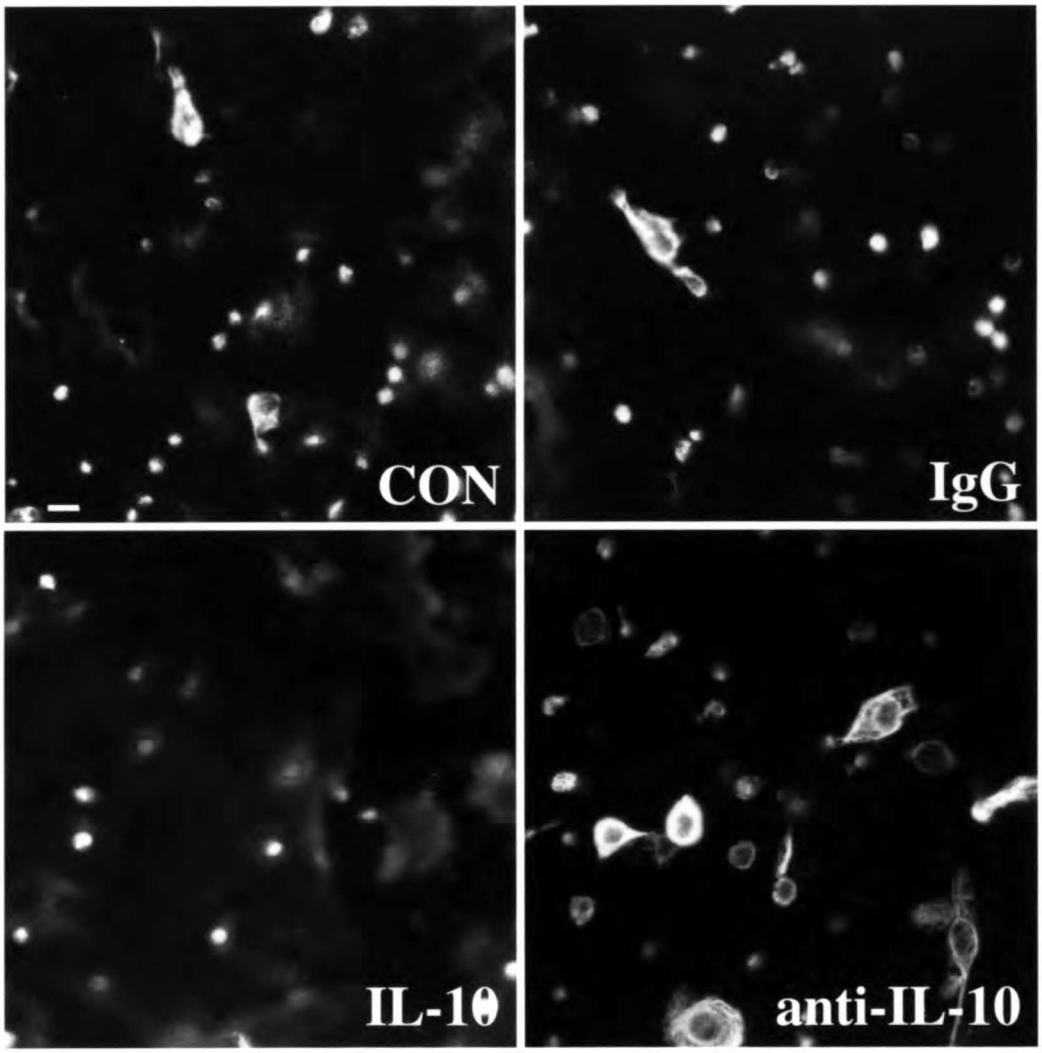


Figure 4-8B

CHAPTER FIVE

Conclusions and Future Directions

The observed immune protection of the allogeneic fetus in mammalian pregnancy has puzzled scientists for decades. Viviparous species have undoubtedly developed complex, redundant mechanisms to avoid maternal immune rejection. As described in Chapter 1, several aspects of human pregnancy are unique to this species, suggesting that distinct protective mechanisms play a role. While the specific ways in which a human fetus is shielded from maternal immune surveillance remain elusive, several important components of the probable mechanism have been identified. These are summarized in Chapter 2, and include the expression of the non-classical class I MHC molecule, HLA-G, by invasive cytotrophoblast cells. Although the precise function of HLA-G has not been identified, recent experimental evidence suggests that this molecule plays a very important role in mediating protective interactions between invasive cytotrophoblasts and maternal decidual leukocytes (Pazmany et al., 1996).

Remaining populations of trophoblast cells, including villous cytotrophoblasts and syncytiotrophoblasts, appear to be null for other known class I or II MHC molecules. Although the possibility remains that these cells express an as-yet-unidentified MHC, the apparent absence of these important antigens leaves invasive cytotrophoblasts susceptible to MHC-unrestricted lysis by NK cells. The lack of classical MHC molecule expression by placental trophoblasts begs scientists to reconsider the transplant analogy often used to describe pregnancy. Mechanisms of graft rejection are now well understood and, as described in Chapter 2, rely on the recognition of genetically foreign class I and II HLA on the surface of grafted cells. Because fetal trophoblast cells do not express classical transplantation antigens, they cannot be compared to a tissue allograft. Unlike organ transplantation, pregnancy is a naturally-occurring phenomenon on which all viviparous animals are dependent for their survival. Clearly, unique and unusual mechanisms must have evolved to allow the coexistence of genetically foreign tissues during gestation.

One of these may involve the secretion of a wide variety of immunomodulatory factors by fetal cells. These may be important in suppressing a potentially harmful maternal immune response. A

large body of work has characterized many immunosuppressive factors in the pregnant human uterus (summarized in Chapter 2), although the majority consists of descriptive observations. Because it is impossible to perform in vivo experiments to study human pregnancy, functional data are difficult to acquire. As another limitation, the precise relationships between the many different cell types in the pregnant human uterus cannot be correctly modeled in vitro. Therefore, the complex regulatory networks between different maternal and fetal cell types are not readily reconstituted in culture systems. To begin to understand these networks, we chose to focus on the production of soluble immunosuppressive factors by a single, purified placental cell type. Human cytotrophoblasts were isolated from placentas of different gestational ages by established methods and cultured on a basement membrane substrate. Under these conditions, we found that undifferentiated, noninvasive cytotrophoblasts secrete the cytokine IL-10 (Chapter 3).

Like all cytokines, IL-10 is a pleiotropic factor whose activities include the inhibition of proinflammatory cytokine synthesis by Th1 cells, macrophages, and NK cells, and the downregulation of class II MHC expression on monocytes/macrophages (Moore et al., 1993). Together, these effects result in the strong suppression of inflammatory, cytotoxic immune responses. We provide evidence in Chapter 3 of this dissertation that cytotrophoblast IL-10 has immunosuppressive activities in vitro, and propose a similar role for cytotrophoblast IL-10 in vivo. IL-10 levels secreted by cytotrophoblasts in vitro are sufficient to inhibit an allogeneic mixed lymphocyte reaction, suggesting that this cytokine may be produced in sufficient quantities in vivo as well. There is evidence that placental cells produce IL-10 at the fetal-maternal interface in vivo. First, we are able to detect mRNA for IL-10 in cytotrophoblasts immediately after purification (see Chapter 3). In addition, we recently showed by immunohistochemistry that cytotrophoblast stem cells in second trimester chorionic villi express IL-10 at the protein level (Figure 5-1). These observations suggest that IL-10 may play an important role in modulating a maternal immune response during human pregnancy in vivo.

Although human pregnancy is not amenable to experimental manipulation, pregnancy disorders are often studied to gain insight into the factors regulating normal placentation. To begin to address the significance of IL-10 production at the maternal-fetal interface, we tested whether cytokine levels were altered in preeclampsia. As described in Chapter 1, this disorder is thought to have an immunological basis and is characterized by deficient cytotrophoblast invasion. Because we have evidence that IL-10 may be involved in both maternal immune suppression (Chapter 3) and the control of cytotrophoblast invasion (Chapter 4), we hypothesized that IL-10 may be disregulated in this disease. IL-10 levels were quantitated by ELISA in the peripheral plasma of preeclamptic patients, as well as age-matched normal pregnant controls. Preliminary evidence suggests that IL-10 may be slightly elevated in preeclamptic pregnancies (Figure 5-2). Few patients suffering from a severe form of preeclampsia complicated by hemolysis, elevated liver enzymes, and low platelet count (HELLP) syndrome experienced a marked elevation in uterine vein plasma IL-10. However, others showed no change in IL-10 levels compared to control subjects. The future quantitation of IL-10 levels in a larger study group will better elucidate the potential role that this cytokine plays in the pathogenesis of preeclampsia.

IL-10 secreted at the maternal-fetal interface may be important in driving the maternal immune system away from a deleterious Th1-mediated cytotoxic response, to a less harmful Th2-directed humoral response. Epidemiological evidence presented in Chapter 2 suggests that the immune system in pregnancy is biased towards a Th2 profile. Further experimental evidence in mice demonstrates that IL-10 plays an important role in directing the development of a humoral response, while preventing cytotoxic reactions after infection with *Listeria monocytogenes* (see Chapter 2). Additional studies in mice show that Th2-type cytokines are differentially expressed at the maternal-fetal interface in vivo (Lin et al., 1993). Whether there is a similar predominance in the pregnant human uterus is unknown. We performed preliminary studies aimed at characterizing the cytokine profile in human pregnancy. In these experiments, mRNA was extracted from biopsies of the placenta, placental bed, and uterine wall opposite the site of placental attachment.

The mRNA for different cytokines, including IL-4, 6, 10, 12 and IFN-γ, were then estimated using a quantitative PCR-based method as described in Chapter 3 (see Figure 3-4). This experimental strategy yielded inconclusive results suggesting that, unlike murine pregnancy, human gestation is not a clearly-defined Th2 phenomenon (data not shown).

We further demonstrate that IL-10 acts to inhibit cytotrophoblast proteinase production and invasion (Chapter 4). While IL-10 has been shown to inhibit MMP-9 production in other cell types, we have described a unique autoregulatory function for this cytokine in the suppression of cytotrophoblast MMP-9 secretion and activity. Although our study did not address the specific cellular mechanisms by which IL-10 downregulates MMP-9 production, important information was provided by the data. We can discern that IL-10's action occurs at a pretranslational level, as steady-state levels of MMP-9 mRNA decrease with a concomitant inhibition of protease secretion. However, we have not determined if the decrease in steady-state MMP-9 message levels is the result of suppressed transcription or shortened half-life.

Engagement of the IL-10 receptor in monocytes and T cells results in tyrosine phosphorylation of the Jak/tyk family of protein kinases, leading to the activation of a group of latent transcription factors known as signal transducers and activators of transcription (STATs; Lai et al., 1996; Weber et al., 1996; Wehinger et al., 1996). STAT proteins then translocate to the nucleus where they bind to common enhancer elements known as GAS (IFN-γ activation sequence) or GRR (IFN-γ response region). Deletion and mutation analyses of the MMP-9 promoter region have identified elements homologous to the binding sites for the transcription factors AP-1, NF-kB and Sp-1 (Huhtala et al., 1991). Because GAS or GRR consensus sequences have not been found, it is unlikely that IL-10 inhibits MMP-9 transcription directly. Future studies will more clearly define the mechanism of suppression of cytotrophoblast MMP-9 gene expression by IL-10.

Because IL-10 inhibits protease production, decreased levels of MMP-9 are available for activation, resulting in reduced invasion. However, the level of MMP-9 activity observed in cytotrophoblast cultures is the net effect of several factors. In addition to the absolute amount of MMP-9 produced, the control of both enzyme activation and TIMP expression are additional targets through which IL-10 can regulate cytotrophoblast proteolytic activity. Unfortunately, we cannot predict the effects of IL-10 on MMP-9 activation as the mechanism of zymogen processing have not yet been elucidated (Sang and Douglas, 1996). We have evidence that cytotrophoblast autoactivation of MMP-9 occurs on the cell surface. To this end, the treatment of CM with rIL-10 and anti–IL-10 in a cell-free system resulted in no change in enzymatic activity (data not shown). With regards to TIMPs, the effects of IL-10 on the expression of different family members are quite variable in distinct cell types (see Chapter 4). We have shown in this report that IL-10 does not regulate the newest member of the TIMP family, TIMP-3, in human cytotrophoblast cells. The downregulation of MMP-9 expression, without a parallel decrease in TIMP-3 production, results in an increase in the inhibitor/proteinase ratio. Thus, MMP-9 activity is additionally suppressed. IL-10 may also directly inhibit MMP-9 activation to downregulate cytotrophoblast invasion.

Our data demonstrate two very important functions for cytotrophoblast IL-10. As a potent immunosuppressive factor, IL-10 may play a role in protecting the fetus from a deleterious maternal immune response. In addition, we show that cytotrophoblast IL-10 is autoregulatory for invasion in vitro, suggesting that invasion of the uterine wall may be controlled by this cytokine in vivo. Together, these very important functions for IL-10 can have profound implications for placental implantation in vivo, thus affecting pregnancy outcome.

Figure 5-1: Villous cytotrophoblasts produce IL-10 in vivo. Sections of a floating chorionic villous were immunostained for IL-10 as described (Abrams, 1995). Individual cytotrophoblast stem cells are seen staining positively for IL-10.

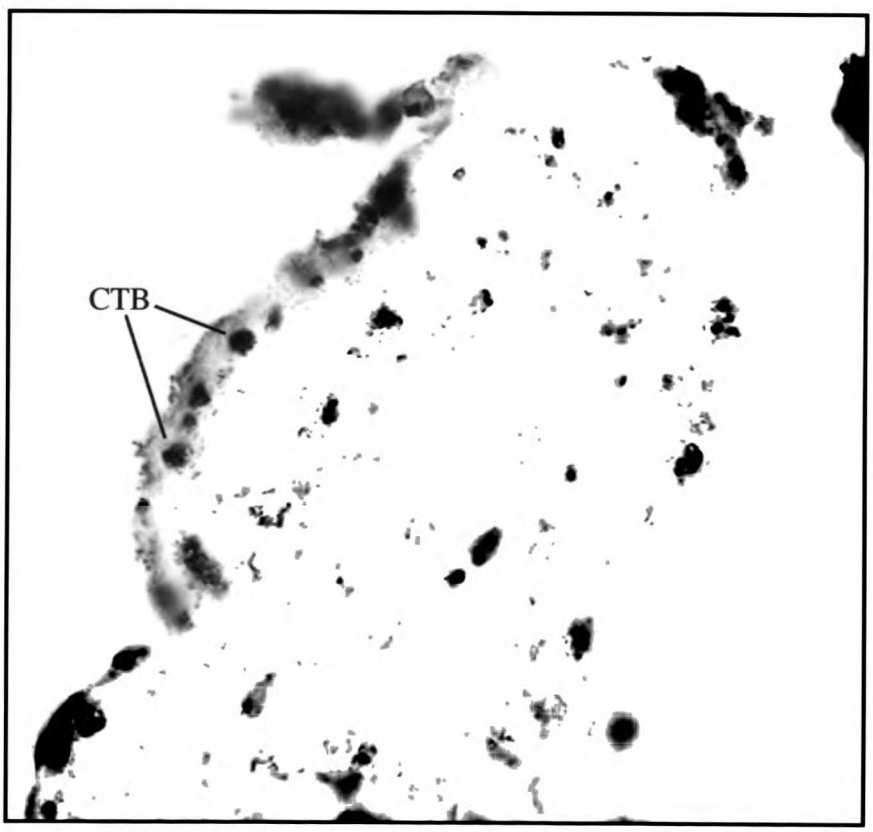


Figure 5-1

Figure 5-2: Circulating levels of IL-10 in preeclampsia. IL-10 in the peripheral plasma from patients suffering from preeclampsia (PE), as well as age-matched normal pregnant (NL) and non-pregnant (NP) controls was quantitated by ELISA. IL-10 levels are slightly elevated in pregnancies complicated by preeclampsia.

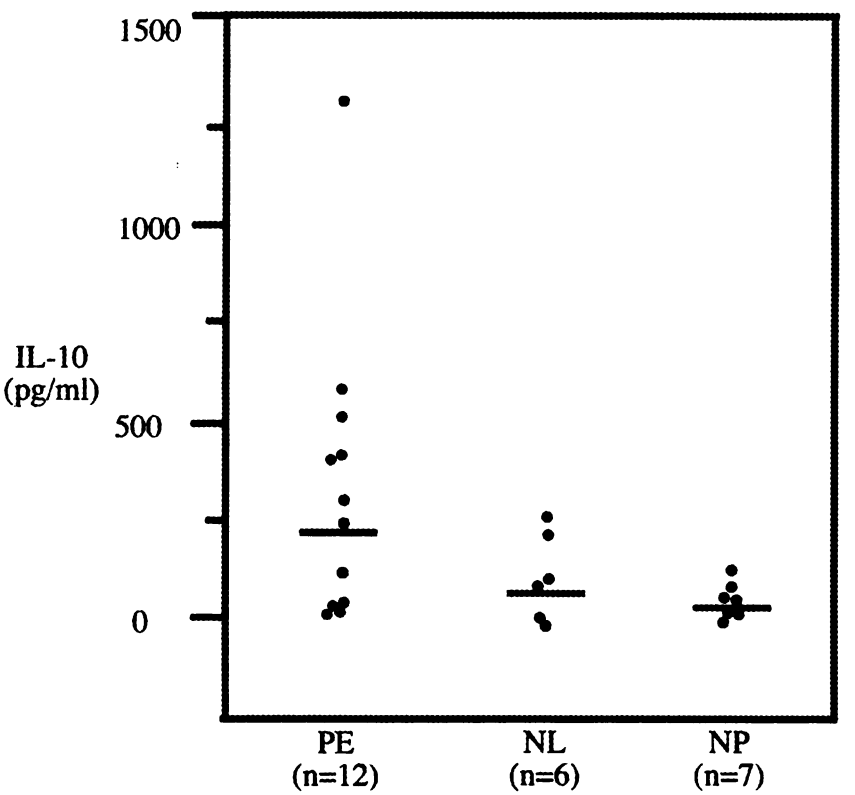


Figure 5-2

REFERENCES

Abbas, A. K., Lichtman, A. H., and Pober, J. S. (1994). Cellular and Molecular Immunology, 2nd Edition (Philadelphia: W.B. Saunders Company).

Abrams, J. S., Roncarolo, M. G., Yssel, H., Andersson, U., Gleich, G. J., and Silver, J. E. (1992). Strategies of anti-cytokine monoclonal antibody development: immunoassay of IL-10 and IL-5 in clinical samples. Immunol Rev 127, 5-24.

Abrams, J. S. (1995). Immunoenzymetric assay of mouse and human cytokines using NIP-labeled anti-cytokine antibodies. In Current Protocols in Immunology, K. Coligan Margulies, Shevach and Strober, ed. (New York: John Wiley & Sons), pp. Unit 6.20.

Abughali, N., Van, der, Kuyp, F, Annable, W., and Kumar, M. L. (1994). Congenital tuberculosis. Pediatr Infect Dis J 13, 738-41.

Adcock, E. 3., Teasdale, T., August, C. S., Cox, S., Meschia, G., Ballaglia, T. C., and Naughton, M. A. (1973). Human chorionic gonadotropin: its possible role in maternal lymphocyte suppression. Science 181, 845-7.

Aksel, S. (1992). Immunologic aspects of reproductive diseases. Jama 268, 2930-4.

Altshuler, G. (1984). Placental infection, and inflammation. In Pathology of the Placenta, E. V. D. K. Perrin, ed. (New York: Churchill Livingstone), pp. 141-163.

Amirhessami-Aghili, N., and Spector, S. A. (1991). Human immunodeficiency virus type 1 infection of human placenta: potential route for fetal infection. J Virol 65, 2231-6.

Andersson, J., Nagy, S., Bjork, L., Abrams, J., Holm, S., and Andersson, U. (1992). Bacterial toxin-induced cytokine production studied at the single-cell level. Immunol Rev 127, 69-96.

Aplin, J. D. (1991). Implantation, trophoblast differentiation and haemochorial placentation: mechanistic evidence in vivo and in vitro. J Cell Sci, 681-92.

Appelbaum, M. G. (1995). Fetal alcohol syndrome: diagnosis, management, and prevention. Nurse Pract 20, 24, 27-8, 31-3 passim.

Apte, S. S., Olsen, B. R., and Murphy, G. (1995). The gene structure of tissue inhibitor of metalloproteinases (TIMP)-3 and its inhibitory activities define the distinct TIMP gene family. J Biol Chem 270, 14313-8.

Arkwright, P., Rademacher, T., Marshall, J., Dwek, R., and Redman, C. (1992). Glycoprotein glycosylation and the immunosuppressive effects of human pregnancy serum. J Reprod Immunol 21, 97-102.

Armitage, R. J., Macduff, B. M., Eisenman, J., Paxton, R., and Grabstein, K. H. (1995). IL-15 has stimulatory activity for the induction of B cell proliferation and differentiation. J Immunol 154, 483-90.

Baldwin, S., and Whitley, R. J. (1989). Intrauterine herpes simplex virus infection. Teratology 39, 1-10.

Barnstable, C. J., Bodmer, W. F., Brown, G., Galfre, G., Milstein, C., Williams, A. F., and Ziegler, A. (1978). Production of monoclonal antibodies to group A erythrocytes, HLA and other human cell surface antigens-new tools for genetic analysis. Cell 14, 9-20.

Beckerman, K. P. (1994). Reproduction and the Immune System. In Basic and Clinical Immunology, D. P. Stites, A. I. Terr and T. G. Parslow, eds. (Norwalk, Connecticut: Appleton & Lange), pp. 552-568.

Beer, A. E., and Sio, J. O. (1982). Placenta as an immunological barrier. Biol Reprod 26, 15-27.

Bejarano, M. T., de Waal Malefyt, R., Abrams, J. S., Bigler, M., Bacchetta, R., de Vries, J., and Roncarolo, M. G. (1992). Interleukin 10 inhibits allogeneic proliferative and cytotoxic T cell responses generated in primary mixed lymphocyte cultures. Int Immunol 4, 1389-97.

Benirschke, K., and Kaufmann, P. (1995). Pathology of the Human Placenta, 3rd Edition (New York: Springer-Verlag New York, Inc.).

Berke, G. (1994). The binding and lysis of target cells by cytotoxic lymphocytes: molecular and cellular aspects. Annu Rev Immunol 12, 735-73.

Berkowitz, R. S., and Goldstein, D. P. (1996). Chorionic tumors. N Engl J Med 335, 1740-8.

Bianchi, D. W. (1995). Prenatal diagnosis by analysis of fetal cells in maternal blood. J Pediatr 127, 847-56.

Billingham, R. E. (1964). Transplantation immunity and the maternal fetal relation. New Eng. J. Med. 270, 667-672, 720-725.

Birkedal-Hansen, H. (1993). Role of cytokines and inflammatory mediators in tissue destruction. J Periodontal Res, 500-10.

Bischof, P., Duberg, S., and Schindler, A. M. (1982). Is pregnancy-associated plasma protein A (PAPP-A) an immunomodulator during human pregnancy? Placenta Suppl 4, 93-102.

Blankenship, T. N., Enders, A. C., and King, B. F. (1993). Trophoblastic invasion and the development of uteroplacental arteries in the macaque: immunohistochemical localization of cytokeratins, desmin, type IV collagen, laminin, and fibronectin. Cell Tissue Res 272, 227-36.

Blyth, B., and Churchill, B. M. (1996). Intersex. In Adult and Pediatric Urology, J. Y. Gillenwater, J. T. Grayhack, S. S. Howards and J. W. Duckett, eds. (St. Louis: Mosby), pp. 2591-2623.

Boehm, K. D., Kelley, M. F., Ilan, J., and Ilan, J. (1989). The interleukin 2 gene is expressed in the syncytiotrophoblast of the human placenta. Proc Natl Acad Sci U S A 86, 656-60.

Borell, U., Fernstroem, I., and Westman, A. (1958). Eine arteriographische Studie des Plazentarkreislaufs. Geburtshilfe Frauenheilkd 18, 1-9.

Borhanmanesh, F., Haghighi, P., Hekmat, K., Rezaizadeh, K., and Ghavami, A. G. (1973). Viral hepatitis during pregnancy. Severity and effect on gestation. Gastroenterology 64, 304-12.

Boyd, J. D., and Hamilton, W. J. (1966). Electron microscopic observations on the cytotrophoblast contribution to the syncytium in the human placenta. J. Anat. 100, 535-548.

Boyd, J. D., and Hamilton, W. J. (1967). Development and structure of the human placenta from the end of the 3rd month of gestation. J Obstet Gynaecol Br Commonw 74, 161-226.

Boyd, J. D., Hamilton, W. J., and Boyd, C. A. (1968). The surface of the syncytium of the human chorionic villus. J. Anat. 102, 553-63.

Boyd, J. D., and Hamilton, W.J. (1970). The Human Placenta (Cambridge: Heffer & Sons).

Brandt, C. D., Rakusan, T. A., Sison, A. V., Saxena, E. S., Ellaurie, M., and Sever, J. L. (1994). Human immunodeficiency virus infection in infants during the first 2 months of life. Reliable detection and evidence of in utero transmission. Arch Pediatr Adolesc Med 148, 250-4.

Breitfeld, P. P., Casanova, J. E., Harris, J. M., Simister, N. E., and Mostov, K. E. (1989). Expression and analysis of the polymeric immunoglobulin receptor in Madin-Darby canine kidney cells using retroviral vectors. Methods Cell Biol 32, 329-37.

Brosens, I., Robertson, W. B., and Dixon, H. G. (1967). The physiological response of the vessels of the placental bed to normal pregnancy. J Pathol Bacteriol 93, 569-79.

Brosens, I. A., Robertson, W. B., and Dixon, H. G. (1972). The role of the spiral arteries in the pathogenesis of preeclampsia. Obstet Gynecol Annu 1, 177-91.

Brosens, I., Robertson, W. B., and Dixon, H. G. (1978). Morphologic changes in uteroplacental arteries during pregnancy. Am J Obstet Gynecol 132, 233.

Bulmer, J. N., and Sunderland, C. A. (1983). Bone-marrow origin of endometrial granulocytes in the early human placental bed. J Reprod Immunol 5, 383-7.

Bulmer, J. N., and Johnson, P. M. (1984). Macrophage populations in the human placenta and amniochorion. Clin Exp Immunol 57, 393-403.

Bulmer, J. N., and Sunderland, C. A. (1984). Immunohistological characterization of lymphoid cell populations in the early human placental bed. Immunology 52, 349-57.

Bulmer, J. N. (1989). Decidual cellular responses. Curr Opin Immunol 1, 1141-7.

Burton, G. J., Mayhew, T. M., and Robertson, L. A. (1989). Stereological re-examination of the effects of varying oxygen tensions on human placental villi maintained in organ culture for up to 12 h. Placenta 10, 263-73.

Cadet, P., Rady, P. L., Tyring, S. K., Yandell, R. B., and Hughes, T. K. (1995). Interleukin-10 messenger ribonucleic acid in human placenta: implications of a role for interleukin-10 in fetal allograft protection. Am J Obstet Gynecol 173, 25-9.

Cantrell, D. (1996). T cell antigen receptor signal transduction pathways. Annu Rev Immunol 14, 259-74.

Casanova, J. E., Apodaca, G., and Mostov, K. E. (1991). An autonomous signal for basolateral sorting in the cytoplasmic domain of the polymeric immunoglobulin receptor. Cell 66, 65-75.

Castellucci, M., Celona, A., Bartels, H., Steininger, B., Benedetto, V., and Kaufmann, P. (1987). Mitosis of the Hofbauer cell: possible implications for a fetal macrophage. Placenta 8, 65-76.

Castellucci, M., Kaufmann, P., and Bischof, P. (1990). Extracellular matrix influences hormone and protein production by human chorionic villi. Cell Tissue Res 262, 135-42.

Cerni, C., Tatra, G., and Bohn, H. (1977). Immunosuppression by human placenta lactogen (HPL) and the pregnancy-specific beta 1-glycoprotein (SP-1). Inhibition of mitogen-induced lymphocyte transformation. Arch Gynakol 223, 1-7.

Chan, J. K., Ng, C. S., and Hui, P. K. (1988). A simple guide to the terminology and application of leucocyte monoclonal antibodies. Histopathology 12, 461-80.

Charbonneau, H., Tonks, N. K., Walsh, K. A., and Fischer, E. H. (1988). The leukocyte common antigen (CD45): a putative receptor-linked protein tyrosine phosphatase. Proc Natl Acad Sci U S A 85, 7182-6.

Chard, T. (1992). Pregnancy tests: a review. Hum Reprod 7, 701-10.

Chasnoff, I. J., Burns, W. J., Schnoll, S. H., and Burns, K. A. (1985). Cocaine use in pregnancy. N Engl J Med 313, 666-9.

Chasnoff, I. J., Griffith, D. R., MacGregor, S., Dirkes, K., and Burns, K. A. (1989). Temporal patterns of cocaine use in pregnancy. Perinatal outcome [see comments]. Jama 261, 1741-4.

Cher, D. J., and Mosmann, T. R. (1987). Two types of murine helper T cell clone. II. Delayed-type hypersensitivity is mediated by TH1 clones. J Immunol 138, 3688-94.

Chien, Y. H., Jores, R., and Crowley, M. P. (1996). Recognition by gamma/delta T cells. Annu Rev Immunol 14, 511-32.

Christmas, S. E., Bulmer, J. N., Meager, A., and Johnson, P. M. (1990). Phenotypic and functional analysis of human CD3- decidual leucocyte clones. Immunology 71, 182-9.

Chun, D., Braga, C., Chow, C., and Lok, L. (1964). Clinical observations on some aspects of hydatidiform moles. J. Obstet. Gynecol. Br. Commonw. 71, 180-184.

Contractor, S. F., and Davies, H. (1973). Effect of human chorionic somatomammotrophin and human chorionic gonadotrophin on phytohaemagglutinin-induced lymphocyte transformation. Nature New Biol 243, 284-6.

Contractor, S. F., Banks, R. W., Jones, C. J., and Fox, H. (1977). A possible role for placental lysosomes in the formation of villous syncytiotrophoblast. Cell Tissue Res 178, 411-9.

Corry, D. B., Folkesson, H. G., Warnock, M. L., Erle, D. J., Matthay, M. A., Wiener, K. J., and Locksley, R. M. (1996). Interleukin 4, but not interleukin 5 or eosinophils, is required in a murine model of acute airway hyperreactivity [see comments]. J Exp Med 183, 109-17.

Cortes, J. E., Talpaz, M., Cabanillas, F., Seymour, J. F., and Kurzrock, R. (1995). Serum levels of interleukin-10 in patients with diffuse large cell lymphoma: lack of correlation with prognosis. Blood 85, 2516-20.

Coutifaris, C., Kao, L. C., Sehdev, H. M., Chin, U., Babalola, G. O., Blaschuk, O. W., and Strauss, J. 3. (1991). E-cadherin expression during the differentiation of human trophoblasts. Development 113, 767-77.

Crane, S., Chun, B., and Acker, D. (1993). Treatment of obstetrical hemorrhagic emergencies. Curr Opin Obstet Gynecol 5, 675-82.

Cresswell, P. (1994). Assembly, transport, and function of MHC class II molecules. Annu Rev Immunol 12, 259-93.

Cross, J. C., Werb, Z., and Fisher, S. J. (1994). Implantation and the placenta: key pieces of the development puzzle. Science 266, 1508-18.

Daiter, E., Pampfer, S., Yeung, Y. G., Barad, D., Stanley, E. R., and Pollard, J. W. (1992). Expression of colony-stimulating factor-1 in the human uterus and placenta. J Clin Endocrinol Metab 74, 850-8.

Damsky, C. H., Fitzgerald, M. L., and Fisher, S. J. (1992). Distribution patterns of extracellular matrix components and adhesion receptors are intricately modulated during first trimester cytotrophoblast differentiation along the invasive pathway, in vivo. J Clin Invest 89, 210-22.

Damsky, C., Sutherland, A., and Fisher, S. (1993). Extracellular matrix 5: adhesive interactions in early mammalian embryogenesis, implantation, and placentation. Faseb J 7, 1320-9.

Damsky, C. H., Librach, C., Lim, K. H., Fitzgerald, M. L., McMaster, M. T., Janatpour, M., Zhou, Y., Logan, S. K., and Fisher, S. J. (1994). Integrin switching regulates normal trophoblast invasion. Development 120, 3657-66.

Daya, S., Clark, D. A., Devlin, C., and Jarrell, J. (1985). Preliminary characterization of two types of suppressor cells in the human uterus. Fertil Steril 44, 778-85.

Daya, S., Clark, D. A., Devlin, C., Jarrell, J., and Chaput, A. (1985). Suppressor cells in human decidua. Am J Obstet Gynecol 151, 267-70.

Daya, S., Johnson, P. M., and Clark, D. A. (1989). Trophoblast induction of suppressor-type cell activity in human endometrial tissue. Am J Reprod Immunol 19, 65-72.

Daya, D., and Sabet, L. (1991). The use of cytokeratin as a sensitive and reliable marker for trophoblastic tissue. Am J Clin Pathol 95, 137-41.

De Andreis, C., Simoni, G., Rossella, F., Castagna, C., Pesenti, E., Porta, G., Colucci, G., Giuntelli, S., Pardi, G., and Semprini, A. E. (1996). HIV-1 proviral DNA polymerase

chain reaction detection in chorionic villi after exclusion of maternal contamination by variable number of tandem repeats analysis. Aids 10, 711-5.

de Waal Malefyt, R., Abrams, J., Bennett, B., Figdor, C. G., and de Vries, J. (1991). Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. J Exp Med 174, 1209-20.

de Waal Malefyt, R., Haanen, J., Spits, H., Roncarolo, M. G., te Velde, A., Figdor, C., Johnson, K., Kastelein, R., Yssel, H., and de Vries, J. (1991). Interleukin 10 (IL-10) and viral IL-10 strongly reduce antigen-specific human T cell proliferation by diminishing the antigen-presenting capacity of monocytes via downregulation of class II major histocompatibility complex expression. J Exp Med 174, 915-24.

De Wolf, F., De Wolf-Peeters, C., and Brosens, I. (1973). Ultrastructure of the spiral arteries in the human placental bed at the end of normal pregnancy. Am J Obstet Gynecol 117, 833-48.

Defilippi, P., Silengo, L., and Tarone, G. (1992). Alpha 6.beta 1 integrin (laminin receptor) is down-regulated by tumor necrosis factor alpha and interleukin-1 beta in human endothelial cells. J Biol Chem 267, 18303-7.

DeGroot, L. J. (1989). Endocrinology (Philadelphia, London, Toronto, Montreal, Sydney, Tokyo: W. B. Saunders Company), pp. 2045-2047.

Delassus, S., Coutinho, G. C., Saucier, C., Darche, S., and Kourilsky, P. (1994). Differential cytokine expression in maternal blood and placenta during murine gestation. J Immunol 152, 2411-20.

Demir, R., Kaufmann, P., Castellucci, M., Erbengi, T., and Kotowski, A. (1989). Fetal vasculogenesis and angiogenesis in human placental villi. Acta Anat (Basel) 136, 190-203.

Denny, L. A., Dehaeck, K., Nevin, J., Soeters, R., van Wijk, A., Megevand, E., and Bloch, B. (1995). Placental site trophoblastic tumor: three case reports and literature review. Gynecol Oncol 59, 300-3.

Desiderio, S. V. (1992). B-cell activation. Curr Opin Immunol 4, 252-6.

Diehl, M., Munz, C., Keilholz, W., Stevanovic, S., Holmes, N., Loke, Y. W., and Rammensee, H. G. (1996). Nonclassical HLA-G molecules are classical peptide presenters. Curr Biol, 6:305-14.

Ding, L., Linsley, P. S., Huang, L. Y., Germain, R. N., and Shevach, E. M. (1993). IL-10 inhibits macrophage costimulatory activity by selectively inhibiting the up-regulation of B7 expression. J Immunol 151, 1224-34.

Dorf, M. E., and Benacerraf, B. (1984). Suppressor cells and immunoregulation. Annu Rev Immunol 2, 127-57.

Douglas, G. C., and King, B. F. (1990). Differentiation of human trophoblast cells in vitro as revealed by immunocytochemical staining of desmoplakin and nuclei. J Cell Sci, 131-41.

Douglas, G. C., and King, B. F. (1992). Maternal-fetal transmission of human immunodeficiency virus: a review of possible routes and cellular mechanisms of infection. Clin Infect Dis, 15(4):678-91.

Douglas, G. C., Fry, G. N., Thirkill, T., Holmes, E., Hakim, H., Jennings, M., and King, B. F. (1991). Cell-mediated infection of human placental trophoblast with HIV in vitro. Aids Res Hum Retroviruses 7, 735-40.

Drutz, D. J., and Catanzaro, A. (1978). Coccidioidomycosis. Part I. Am Rev Respir Dis 117, 559-85.

Dungy, L. J., Siddiqi, T. A., and Khan, S. (1991). Transforming growth factor-beta 1 expression during placental development. Am J Obstet Gynecol, 853-7.

Durez, P., Abramowicz, D., Gerard, C., Van Mechelen, M., Amraoui, Z., Dubois, C., Leo, O., Velu, T., and Goldman, M. (1993). In vivo induction of interleukin 10 by anti-CD3 monoclonal antibody or bacterial lipopolysaccharide: differential modulation by cyclosporin A. J Exp Med 177, 551-5.

Ellis, S. A., Sargent, I. L., Redman, C. W., and McMichael, A. J. (1986). Evidence for a novel HLA antigen found on human extravillous trophoblast and a choriocarcinoma cell line. Immunology 59, 595-601.

Ellis, S. A., Palmer, M. S., and McMichael, A. J. (1990). Human trophoblast and the choriocarcinoma cell line BeWo express a truncated HLA Class I molecule. J Immunol 144, 731-5.

Enders, A. E. (1965). A comparative study of the fine structure of the trophoblast in several hemochorial placentas. Am. J. Anat. 116, 29-68.

Enders, A. C., and Schlafke, S. (1969). Cytological aspects of trophoblast-uterine interaction in early implantation. Am J Anat 125, 1-29.

Enders, A. C., Hendrickx, A. G., and Schlafke, S. (1983). Implantation in the rhesus monkey: initial penetration of endometrium. Am J Anat 167, 275-98.

Erbach, G. T., Semple, J. P., Milford, E., Goguen, J., Osathanondh, R., and Kurnick, J. T. (1993). Phenotypic characteristics of lymphocyte populations isolated from middle gestation human placenta. J Reprod Immunol 25, 1-13.

Evans, H. E., and Frenkel, L. D. (1994). Congenital syphilis. Clin Perinatol 21, 149-62.

Faulk, W. P., and Temple, A. (1976). Distribution of beta2 microglobulin and HLA in chorionic villi of human placentae. Nature 262, 799-802.

Faulk, W. P., Sanderson, A. R., and Temple, A. (1977). Distribution of MHC antigens in human placental chorionic villi. Transplant Proc 9, 1379-84.

Faulk, W. P., and Hunt, J. S. (1989). Human placentae: view from an immunological bias. Am J Reprod Immunol 21, 108-13.

Fearon, D. T., and Locksley, R. M. (1996). The instructive role of innate immunity in the acquired immune response. Science 272, 50-3.

Ferrara, J. L., and Deeg, H. J. (1991). Graft-versus-host disease [see comments]. N Engl J Med 324, 667-74.

Ferry, B. L., Starkey, P. M., Sargent, I. L., Watt, G. M., Jackson, M., and Redman, C. W. (1990). Cell populations in the human early pregnancy decidua: natural killer activity and response to interleukin-2 of CD56-positive large granular lymphocytes. Immunology 70, 446-52.

Fiorentino, D. F., Bond, M. W., and Mosmann, T. R. (1989). Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. J Exp Med 170, 2081-95.

Fisher, S. J., Cui, T. Y., Zhang, L., Hartman, L., Grahl, K., Zhang, G. Y., Tarpey, J., and Damsky, C. H. (1989). Adhesive and degradative properties of human placental cytotrophoblast cells in vitro. J Cell Biol 109, 891-902.

Fisher, S. J., and Damsky, C. H. (1993). Human cytotrophoblast invasion. Semin Cell Biol 4, 183-8.

Flynn, A., Finke, J. H., and Hilfiker, M. L. (1982). Placental mononuclear phagocytes as a source of interleukin-1. Science 218, 475-7.

Ford, J. H., Brown, J. K., Lew, W. Y., and Peters, G. B. (1986). Diploid complete hydatidiform mole, mosaic for normally fertilized cells and androgenetic homozygous cells. Case report. Br J Obstet Gynaecol 93, 1181-6.

Friedman, S. A., Taylor, R. N., and Roberts, J. M. (1991). Pathophysiology of preeclampsia. Clin Perinatol 18, 661-82.

Garcia-Lloret, M., Morrish, D. W., Wegmann, T. G., Honore, L., Turner, A. R., and Guilbert, L. J. (1994). Demonstration of functional cytokine-placental interactions: CSF-1 and GM-CSF stimulate human cytotrophoblast differentiation and peptide hormone secretion. Exp Cell Res 214, 46-54.

Garland, S. M., and O'Reilly, M. A. (1995). The risks and benefits of antimicrobial therapy in pregnancy. Drug Saf 13, 188-205.

Gastl, G. A., Abrams, J. S., Nanus, D. M., Oosterkamp, R., Silver, J., Liu, F., Chen, M., Albino, A. P., and Bander, N. H. (1993). Interleukin-10 production by human carcinoma cell lines and its relationship to interleukin-6 expression. Int J Cancer 55, 96-101.

Gehrz, R. C., Christianson, W. R., Linner, K. M., Conroy, M. M., McCue, S. A., and Balfour, H. J. (1981). A longitudinal analysis of lymphocyte proliferative responses to mitogens and antigens during human pregnancy. Am J Obstet Gynecol 140, 665-70.

Genbacev, O., Bass, K. E., Joslin, R. J., and Fisher S. J. (1995). Maternal smoking inhibits early human cytotrophoblast differentiation. Reprod. Toxicol. 9(3):245-55.

Genbacev, O., Joslin, R., Damsky, C. H., Polliotti, B. M., and Fisher, S. J. (1996). Hypoxia alters early gestation human cytotrophoblast differentiation/invasion in vitro and models the placental defects that occur in preeclampsia. J Clin Invest 97, 540-50.

Geraghty, D. E., Koller, B. H., and Orr, H. T. (1987). A human major histocompatibility complex class I gene that encodes a protein with a shortened cytoplasmic segment. Proc Natl Acad Sci U S A 84, 9145-9.

Gerretsen, G., Huisjes, H. J., and Elema, J. D. (1981). Morphological changes of the spiral arteries in the placental bed in relation to pre-eclampsia and fetal growth retardation. Br J Obstet Gynaecol 88, 876-81.

Ghildyal, N., McNeil, H. P., Stechschulte, S., Austen, K. F., Silberstein, D., Gurish, M. F., Somerville, L. L., and Stevens, R. L. (1992). IL-10 induces transcription of the gene for mouse mast cell protease-1, a serine protease preferentially expressed in mucosal mast cells of Trichinella spiralis-infected mice. J Immunol 149, 2123-9.

Ghildyal, N., McNeil, H. P., Gurish, M. F., Austen, K. F., and Stevens, R. L. (1992). Transcriptional regulation of the mucosal mast cell-specific protease gene, MMCP-2, by interleukin 10 and interleukin 3. J Biol Chem 267, 8473-7.

Gill, T. 3., and Repetti, C. F. (1979). Immunologic and genetic factors influencing reproduction. A review. Am J Pathol 95, 465-570.

Goldstein, J., Braverman, M., Salafia, C., and Buckley, P. (1988). The phenotype of human placental macrophages and its variation with gestational age. Am J Pathol 133, 648-59.

Goodfellow, P. N., Barnstable, C. J., Bodmer, W. F., Snary, D., and Crumpton, M. J. (1976). Expression of HLA system antigens on placenta. Transplantation 22, 595-603.

Goodnow, C. C. (1996). Balancing immunity and tolerance: deleting and tuning lymphocyte repertoires. Proc Natl Acad Sci U S A 93, 2264-71.

Gosseye, S., and Fox, H. (1984). An immunohistological comparison of the secretory capacity of villous and extravillous trophoblast in the human placenta. Placenta 5, 329-47.

Grannum, P. A., Copel, J. A., Moya, F. R., Scioscia, A. L., Robert, J. A., Winn, H. N., Coster, B. C., Burdine, C. B., and Hobbins, J. C. (1988). The reversal of hydrops fetalis by intravascular intrauterine transfusion in severe isoimmune fetal anemia. Am J Obstet Gynecol 158, 914-9.

Greenspoon, J. S., and Settlage, R. H. (1989). Isolation of human immunodeficiency virus from the placenta or from maternal blood contaminating the placenta? [letter; comment]. Am J Obstet Gynecol 161, 501-2.

Guilbert, L., Robertson, S. A., and Wegmann, T. G. (1993). The trophoblast as an integral component of a macrophage-cytokine network. Immunol Cell Biol, 49-57.

Gumperz, J. E., and Parham, P. (1995). The enigma of the natural killer cell. Nature 378, 245-8.

Hamilton, W. J., and Boyd, J. D. (1960). Development of the human placenta in the first three months of gestation. J. Anat. 94, 297-328.

Hamilton, W. J., and Boyd, J. D. (1966). Trophoblast in human utero-placental arteries. Nature 212, 906-8.

Hamilton, W. J., and Boyd, J. D. (1966). Specializations of the syncytium of the human chorion. Br Med J 5502, 1501-6.

Hertig, A. T., and Rock, J. (1941). Two human ova of the previllous stage having an ovulation age of about eleven and twelve days respectively. Contrib. Embryol. Carnegie Inst. 29, 127-156.

Hertig, A. T., and Rock, J. (1945). Two human ova of the previllous stage, having a developmental age of about seven and nine days respectively. Contrib. Embryol. Carnegie Inst. 31, 65-84.

Hertig, A. T., and Rock, J. (1949). Two human ova of the previllous stage, having a developmental age of about eight and nine days respectively. Contrib. Embryol. Carnegie Inst. 33, 169-186.

Hertig, A. T., Rock, J., and Adams, E. C. (1956). A description of 34 human ova within the first seventeen days of development. Am. J. Anat. 98, 435.

Hertig, A. T., and Rock, J. (1973). Searching for early fertilized human ova. Gynecol Invest 4, 121-39.

Heyborne, K. D., Cranfill, R. L., Carding, S. R., Born, W. K., and O'Brien, R. L. (1992). Characterization of gamma delta T lymphocytes at the maternal-fetal interface. J Immunol 149, 2872-8.

Heyborne, K. D., McGregor, J. A., Henry, G., Witkin, S. S., and Abrams, J. S. (1994). Interleukin-10 in amniotic fluid at midtrimester: immune activation and suppression in relation to fetal growth. Am J Obstet Gynecol 171, 55-9.

Heyborne, K., Fu, Y. X., Nelson, A., Farr, A., O'Brien, R., and Born, W. (1994). Recognition of trophoblasts by gamma delta T cells. J Immunol 153, 2918-26.

Hill, J. A., Polgar, K., and Anderson, D. J. (1995). T-helper 1-type immunity to trophoblast in women with recurrent spontaneous abortion [see comments]. Jama 273, 1933-6.

Hillier, S. L., Martius, J., Krohn, M., Kiviat, N., Holmes, K. K., and Eschenbach, D. A. (1988). A case-control study of chorioamnionic infection and histologic chorioamnionitis in prematurity. N Engl J Med 319, 972-8.

Hofbauer, J. (1903). Über das konstante Vorkommen bisher unbekannter zelliger Formelemente in der Chorionzotte der menschlichen Plazenta und über Embryotrophe. Wien. Klin. Wochenschr. 16, 871-873.

Holmes, C. H., Simpson, K. L., Okada, H., Okada, N., Wainwright, S. D., Purcell, D. F., and Houlihan, J. M. (1992). Complement regulatory proteins at the feto-maternal interface during human placental development: distribution of CD59 by comparison with membrane cofactor protein (CD46) and decay accelerating factor (CD55). Eur J Immunol 22, 1579-85.

Holzgreve, W., Ganshirt, A. D., Burschyk, M., Horst, J., Miny, P., Gal, A., and Pohlschmidt, M. (1990). Detection of fetal DNA in maternal blood by PCR [letter; comment]. Lancet 335, 1220-1.

Hormann, G., Herbst, R., and Ullmann, G. (1969). [Transformation of the cytotrophoblast into the syncytiotrophoblast]. Z Geburtshilfe Perinatol 171, 172-81.

Hoshina, M., Boothby, M., and Boime, I. (1982). Cytological localization of chorionic gonadotropin alpha and placental lactogen mRNAs during development of the human placenta. J Cell Biol 93, 190-8.

Howard, M., and O'Garra, A. (1992). Biological properties of interleukin 10. Immunol Today 13, 198-200.

Howard, M. C., Miyajima, A., and Coffman, R. (1993). T-Cell-Derived Cytokines and Their Receptors. In Fundamental Immunology, W. E. Paul, ed. (New York: Raven Press, Ltd.), pp. 763-800.

Hsi, B. L., Yeh, C. J., and Faulk, W. P. (1984). Class I antigens of the major histocompatibility complex on cytotrophoblast of human chorion laeve. Immunology 52, 621-9.

Huhtala, P., Tuuttila, A., Chow, L. T., Lohi, J., Keski-Oja, J., and Tryggvason, K. (1991). Complete structure of the human gene for 92-kDa type IV collagenase. Divergent regulation of expression for the 92- and 72-kilodalton enzyme genes in HT-1080 cells. J Biol Chem, 266:16485-90.

Hulbert, T. V. (1992). Congenital malaria in the United States: report of a case and review. Clin Infect Dis 14, 922-6.

Hunt, J. S., King, C. J., and Wood, G. W. (1984). Evaluation of human chorionic trophoblast cells and placental macrophages as stimulators of maternal lymphocyte proliferation in vitro. J Reprod Immunol 6, 377-91.

Hunt, J. S., Andrews, G. K., Fishback, J. L., Feess, M., and Wood, G. W. (1988). Amnion membrane epithelial cells express class I HLA and contain class I HLA mRNA. J Immunol 140, 2790-5.

Hunt, J. S. (1989). Cytokine networks in the uteroplacental unit: macrophages as pivotal regulatory cells. J Reprod Immunol 16, 1-17.

Hunt, J. S., Fishback, J. L., Chumbley, G., and Loke, Y. W. (1990). Identification of class I MHC mRNA in human first trimester trophoblast cells by in situ hybridization. J Immunol 144, 4420-5.

Hunt, J. S., and Pollard, J. W. (1992). Macrophages in the uterus and placenta. Curr Top Microbiol Immunol 181, 39-63.

Hunziker, R. D., and Wegmann, T. G. (1986). Placental immunoregulation. Crit Rev Immunol 6, 245-85.

Inman, W., Pearce, G., and Wilton, L. (1994). Safety of fluconazole in the treatment of vaginal candidiasis. A prescription-event monitoring study, with special reference to the outcome of pregnancy. Eur J Clin Pharmacol 46, 115-8.

Itohara, S., Farr, A. G., Lafaille, J. J., Bonneville, M., Takagaki, Y., Haas, W., and Tonegawa, S. (1990). Homing of a gamma delta thymocyte subset with homogeneous T-cell receptors to mucosal epithelia. Nature 343, 754-7.

Jokhi, P. P., King, A., Boocock, C., and Loke, Y. W. (1995). Secretion of colony stimulating factor-1 by human first trimester placental and decidual cell populations and the effect of this cytokine on trophoblast thymidine uptake in vitro. Hum Reprod 10, 2800-7.

June, C. H., Ledbetter, J. A., Linsley, P. S., and Thompson, C. B. (1990). Role of the CD28 receptor in T-cell activation. Immunol Today 11, 211-6.

Kabawat, S. E., Mostoufi, Z. M., Driscoll, S. G., and Bhan, A. K. (1985). Implantation site in normal pregnancy. A study with monoclonal antibodies. Am J Pathol 118, 76-84.

Kagi, D., Ledermann, B., Burki, K., Zinkernagel, R. M., and Hengartner, H. (1996). Molecular mechanisms of lymphocyte-mediated cytotoxicity and their role in immunological protection and pathogenesis in vivo. Annu Rev Immunol 14, 207-32.

Kaiser, R. (1960). Über Rückbildungsvorgaenge in der Dezidua während der Schwangerschaft. Arch. Gynecol. 192, 209-220.

Kajii, T., and Ohama, K. (1977). Androgenetic origin of hydatidiform mole. Nature 268, 633-4.

Kameda, T., Matsuzaki, N., Sawai, K., Okada, T., Saji, F., Matsuda, T., Hirano, T., Kishimoto, T., and Tanizawa, O. (1990). Production of interleukin-6 by normal human trophoblast. Placenta 11, 205-13.

Kanzaki, H., Yui, J., Iwai, M., Imai, K., Kariya, M., Hatayama, H., Mori, T., Guilbert, L. J., and Wegmann, T. G. (1992). The expression and localization of mRNA for colony-stimulating factor (CSF)-1 in human term placenta. Hum Reprod 7, 563-7.

Kaufmann, P., and Stegner, H. E. (1972). [Functional differentiation of the human placental syncytiotrophoblast]. Z Zellforsch Mikrosk Anat 135, 361-82.

Kaufmann, P. (1972). [Observations on the Langhans cells in the human placenta]. Z Zellforsch Mikrosk Anat 128, 283-302.

Kaufmann, P., Stark, J., and Stegner, H. E. (1977). The villous stroma of the human placenta. I. The ultrastructure of fixed connective tissue cells. Cell Tissue Res 177, 105-21.

Kauma, S., Matt, D., Strom, S., Eierman, D., and Turner, T. (1990). Interleukin-1 beta, human leukocyte antigen HLA-DR alpha, and transforming growth factor-beta expression in endometrium, placenta, and placental membranes. Am J Obstet Gynecol, 1430-7.

Kauma, S. W., Aukerman, S. L., Eierman, D., and Turner, T. (1991). Colony-stimulating factor-1 and c-fms expression in human endometrial tissues and placenta during the menstrual cycle and early pregnancy. J Clin Endocrinol Metab 73, 746-51.

Kim, C. K., and Benirschke, K. (1971). Autoradiographic study of the "X cells" in the human placenta. Am J Obstet Gynecol 109, 96-102.

King, A., Wellings, V., Gardner, L., and Loke, Y. W. (1989). Immunocytochemical characterization of the unusual large granular lymphocytes in human endometrium throughout the menstrual cycle. Hum Immunol 24, 195-205.

King, A., Birkby, C., and Loke, Y. W. (1989). Early human decidual cells exhibit NK activity against the K562 cell line but not against first trimester trophoblast. Cell Immunol 118, 337-44.

King, A., and Loke, Y. W. (1991). On the nature and function of human uterine granular lymphocytes [see comments]. Immunol Today 12, 432-5.

Kleihauer, E., Braun, H., and Betke, K. (1957). Demonstration von fetalem Hämoglobin in den Erythrocyten eines Blautausstriches. Klin. Wochenschr. 35, 637-638.

Klein, J., Takahata, N., and Ayala, F. J. (1993). MHC polymorphism and human origins. Sci Am 269, 78-83.

Kliman, H. J., Nestler, J. E., Sermasi, E., Sanger, J. M., and Strauss, J. 3. (1986). Purification, characterization, and in vitro differentiation of cytotrophoblasts from human term placentae. Endocrinology 118, 1567-82.

Kohler, P. O., and Bridson, W. E. (1971). Isolation of hormone-producing clonal lines of human choriocarcinoma. J Clin Endocrinol Metab 32, 683-7.

Korzeniowski, O. M. (1995). Antibacterial agents in pregnancy. Infect Dis Clin North Am 9, 639-51.

Kovar, I. Z., and Riches, P. G. (1988). C3 and C4 complement components and acute phase proteins in late pregnancy and parturition. J Clin Pathol 41, 650-2.

Kovats, S., Main, E. K., Librach, C., Stubblebine, M., Fisher, S. J., and DeMars, R. (1990). A class I antigen, HLA-G, expressed in human trophoblasts. Science 248, 220-3.

Kristoffersen, E. K. (1996). Human placental Fc gamma-binding proteins in the maternofetal transfer of IgG. Apmis Suppl 64, 5-36.

Kronenberg, M. (1994). Antigens recognized by gamma delta T cells. Curr Opin Immunol, 6:64-71.

Kruisbeek, A. M., and Amsen, D. (1996). Mechanisms underlying T-cell tolerance. Curr Opin Immunol 8, 233-44.

Kuhn, R., Lohler, J., Rennick, D., Rajewsky, K., and Muller, W. (1993). Interleukin-10-deficient mice develop chronic enterocolitis [see comments]. Cell 75, 263-74.

Lacraz, S., Nicod, L., Galve de Rochemonteix, B., Baumberger, C., Dayer, J. M., and Welgus, H. G. (1992). Suppression of metalloproteinase biosynthesis in human alveolar macrophages by interleukin-4. J Clin Invest 90, 382-8.

Lacraz, S., Nicod, L. P., Chicheportiche, R., Welgus, H. G., and Dayer, J. M. (1995). IL-10 inhibits metalloproteinase and stimulates TIMP-1 production in human mononuclear phagocytes. J Clin Invest 96, 2304-10.

Lai, C. F., Ripperger, J., Morella, K. K., Jurlander, J., Hawley, T. S., Carson, W. E., Kordula, T., Caligiuri, M. A., Hawley, R. G., Fey, G. H., and Baumann, H. (1996). Receptors for interleukin (IL)-10 and IL-6-type cytokines use similar signaling mechanisms for inducing transcription through IL-6 response elements. J Biol Chem 271, 13968-75.

Lanier, L. L., and Phillips, J. H. (1996). Inhibitory MHC class I receptors on NK cells and T cells. Immunol Today 17, 86-91.

Lata, J. A., Tuan, R. S., Shepley, K. J., Mulligan, M. M., Jackson, L. G., and Smith, J. B. (1992). Localization of major histocompatibility complex class I and II mRNA in human first-trimester chorionic villi by in situ hybridization. J Exp Med 175, 1027-32.

Leach, J. L., Sedmak, D. D., Osborne, J. M., Rahill, B., Lairmore, M. D., and Anderson, C. L. (1996). Isolation from human placenta of the IgG transporter, FcRn, and localization to the syncytiotrophoblast: implications for maternal-fetal antibody transport. J Immunol 157, 3317-22.

Leahy, D. J. (1995). A structural view of CD4 and CD8. Faseb J 9, 17-25.

Lehmann, A. K., Halstensen, A., Sornes, S., Rokke, O., and Waage, A. (1995). High levels of interleukin 10 in serum are associated with fatality in meningococcal disease. Infect Immun 63, 2109-12.

Librach, C. L., Werb, Z., Fitzgerald, M. L., Chiu, K., Corwin, N. M., Esteves, R. A., Grobelny, D., Galardy, R., Damsky, C. H., and Fisher, S. J. (1991). 92-kD type IV collagenase mediates invasion of human cytotrophoblasts. J Cell Biol 113, 437-49.

Librach, C. L., Feigenbaum, S. L., Bass, K. E., Cui, T. Y., Verastas, N., Sadovsky, Y., Quigley, J. P., French, D. L., and Fisher, S. J. (1994). Interleukin-1 beta regulates human cytotrophoblast metalloproteinase activity and invasion in vitro. J Biol Chem 269, 17125-31.

Lim, K. H., and Friedman, S. A. (1993). Hypertension in pregnancy. Curr Opin Obstet Gynecol 5, 40-9.

Lim, K.-H., Zhou, Y., Janatpour, M. J., McMaster, M., Bass, K., Chun, S.-H., and Fisher, S. J. (1997). Human cytotrophoblast differentiation/invasion is abnormal in preeclampsia. Developmental Biology (Submitted).

Lin, H., Mosmann, T. R., Guilbert, L., Tuntipopipat, S., and Wegmann, T. G. (1993). Synthesis of T helper 2-type cytokines at the maternal-fetal interface. J Immunol 151, 4562-73.

Lindenberg, S., Nielsen, M. H., and Lenz, S. (1985). In vitro studies of human blastocyst implantation. Ann N Y Acad Sci 442, 368-74.

Lindenberg, S., Hyttel, P., Lenz, S., and Holmes, P. V. (1986). Ultrastructure of the early human implantation in vitro. Hum Reprod 1, 533-8.

Lindenberg, S., Hyttel, P., Sjogren, A., and Greve, T. (1989). A comparative study of attachment of human, bovine and mouse blastocysts to uterine epithelial monolayer. Hum Reprod 4, 446-56.

Lindheimer, M. D., and Katz, A. I. (1989). Preeclampsia: pathophysiology, diagnosis, and management. Annu Rev Med 40, 233-50.

Lindoff, C. and Astedt, B. (1994). Plasminogen activator of urokinase type and its inhibitor of placental type in hypertensive pregnancies and in intrauterine growth retardation: possible markers of placental function. Am J Obstet Gynecol, 171(1):60-4.

Little, C. C. (1924). The genetics of tissue transplantation in mammals. J. Cancer Res., 75-95.

Ljunggren, H. G., and Karre, K. (1990). In search of the 'missing self': MHC molecules and NK cell recognition [see comments]. Immunol Today 11, 237-44.

Lloyd, L. K., Miya, F., Hebertson, R. M., Kochenour, N. K., and Scott, J. R. (1980). Intrapartum fetomaternal bleeding in Rh-negative women. Obstet Gynecol 56, 285-8.

Locksley, R. M., and Scott, P. (1991). Helper T-cell subsets in mouse leishmaniasis: induction, expansion and effector function. Immunol Today 12, A58-61.

Loke, Y. W., and King, A. (1991). Recent developments in the human maternal-fetal immune interaction. Curr Opin Immunol 3, 762-6.

Lopez-Llera, M. (1992). Main clinical types and subtypes of eclampsia [see comments]. Am J Obstet Gynecol, 4-9.

Luft, B. J., and Remington, J. S. (1984). The adverse effect of pregnancy on macrophage activation. Cell Immunol 85, 94-9.

Lunell, N. O., Nylund, L. E., Lewander, R., and Sarby, B. (1982). Uteroplacental blood flow in pre-eclampsia measurements with indium-113m and a computer-linked gamma camera. Clin Exp Hypertens [b] 1, 105-17.

Lyons, J. G., Birkedal, H. B., Pierson, M. C., Whitelock, J. M., and Birkedal, H. H. (1993). Interleukin-1 beta and transforming growth factor-alpha/epidermal growth factor induce expression of M(r) 95,000 type IV collagenase/gelatinase and interstitial fibroblast-type collagenase by rat mucosal keratinocytes. J Biol Chem 268, 19143-51.

Mabie, W. C. (1992). Placenta previa. Clin. Perinatol. 19(2):425-35.

Manaseki, S., and Searle, R. F. (1989). Natural killer (NK) cell activity of first trimester human decidua. Cell Immunol 121, 166-73.

Marcus, G. J., and Shelesnyak, M. C. (1968). Studies on the mechnism of nidation. XXXII Coital elevation of uterine histamine content. Acta. Endocrinol. 57, 136-141.

Martin, J. J., Sessums, J. K., Martin, R. W., Pryor, J. A., and Morrison, J. C. (1988). Abdominal pregnancy: current concepts of management. Obstet Gynecol 71, 549-57.

Martinoli, C., Castellucci, M., Zaccheo, D., and Kaufmann, P. (1984). Scanning electron microscopy of stromal cells of human placental villi throughout pregnancy. Cell Tissue Res 235, 647-55.

Matrisian, L. M. (1990). Metalloproteinases and their inhibitors in matrix remodeling. Trends Genet 6, 121-5.

Matsui, D. (1994). Prevention, diagnosis, and treatment of fetal toxoplasmosis. Clin Perinatol 21, 675-89.

Matthes, T., Werner, F. C., Tang, H., Zhang, X., Kindler, V., and Zubler, R. H. (1993). Cytokine mRNA expression during an in vitro response of human B lymphocytes: kinetics

of B cell tumor necrosis factor alpha, interleukin (IL)6, IL-10, and transforming growth factor beta 1 mRNAs. J Exp Med 178, 521-8.

McCracken, S., Layton, J. E., Shorter, S. C., Starkey, P. M., Barlow, D. H., and Mardon, H. J. (1996). Expression of granulocyte-colony stimulating factor and its receptor is regulated during the development of the human placenta. J Endocrinol 149, 249-58.

McIntyre, J. A., and Faulk, W. P. (1986). Trophoblast antigens in normal and abnormal human pregnancy. Clin Obstet Gynecol 29, 976-98.

McMaster, M. T., Librach, C. L., Zhou, Y., Lim, K. H., Janatpour, M. J., DeMars, R., Kovats, S., Damsky, C., and Fisher, S. J. (1995). Human placental HLA-G expression is restricted to differentiated cytotrophoblasts. J Immunol 154, 3771-8.

Medawar, P. B. (1954). Some immunological and endocrinological problems raised by the evolution of viviparity in vertebrates. Symp. Soc. Exp. Biol. 7, 320-328.

Melchers, F., Rolink, A., Grawunder, U., Winkler, T. H., Karasuyama, H., Ghia, P., and Andersson, J. (1995). Positive and negative selection events during B lymphopoiesis. Curr Opin Immunol 7, 214-27.

Menu, E., David, V., Bensussan, A., and Chaouat, G. (1989). Immunoactive products of human placenta. II. Direct inhibition of non-MHC restricted cytolytic activity of human CD3 alpha-beta but not CD3 gamma-delta expressing T cell clones. J Reprod Immunol 16, 137-50.

Mertz, P. M., DeWitt, D. L., Stetler-Stevenson, W. G., and Wahl, L. M. (1994). Interleukin 10 suppression of monocyte prostaglandin H synthase-2. Mechanism of inhibition of prostaglandin-dependent matrix metalloproteinase production. J Biol Chem 269, 21322-9.

Metcalfe, J., Romney, S. L., and Ramsey, L. H. (1955). Estimation of uterine blood flow in normal human pregnancy at term. J. Clin. Invest. 34, 1632-1638.

Michie, C. A., and Hyer, W. (1995). When does HIV cross the placenta? [letter]. Lancet 345, 517-8.

Mincheva-Nilsson, L., Hammarstrom, S., and Hammarstrom, M. L. (1992). Human decidual leukocytes from early pregnancy contain high numbers of gamma delta+ cells and show selective down-regulation of alloreactivity. J Immunol 149, 2203-11.

Mincheva-Nilsson, L., Baranov, V., Yeung, M. M., Hammarstrom, S., and Hammarstrom, M. L. (1994). Immunomorphologic studies of human decidua-associated lymphoid cells in normal early pregnancy. J Immunol 152, 2020-32.

Mittendorf, R., and Williams, M. A. (1991). Rho(D) immunoglobulin (RhoGAM): how it came into being [see comments]. Obstet Gynecol 77, 301-3.

Moore, K. W., O'Garra, A., de Waal Malefyt, R., Vieira, P., and Mosmann, T. R. (1993). Interleukin-10. Annu Rev Immunol 11, 165-90.

Moore, K. L., and Persaud, T. V. N. (1993). The Developing Human, 5th Edition (Philadelphia: W.B. Saunders Company).

Moretta, L., Ciccone, E., Ferrini, S., Pelicci, P. G., Mingari, M. C., Zeromski, J., Bottino, C., Grossi, C., and Moretta, A. (1991). Molecular and cellular analysis of human T lymphocytes expressing gamma delta T-cell receptor. Immunol Rev 120, 117-35.

Moretta, L., Ciccone, E., Moretta, A., Hoglund, P., Ohlen, C., and Karre, K. (1992). Allorecognition by NK cells: nonself or no self? Immunol Today 13, 300-6.

Moretta, A., Bottino, C., Vitale, M., Pende, D., Biassoni, R., Mingari, M. C., and Moretta, L. (1996). Receptors for HLA class-I molecules in human natural killer cells. Annu Rev Immunol 14, 619-48.

Morii, T., Nishikawa, K., Saito, S., Enomoto, M., Ito, A., Kurai, N., Shimoyama, T., Ichijo, M., and Narita, N. (1993). T-cell receptors are expressed but down-regulated on intradecidual T lymphocytes [see comments]. Am J Reprod Immunol 29, 1-4.

Mosmann, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A., and Coffman, R. L. (1986). Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J Immunol 136, 2348-57.

Mosmann, T. R., and Coffman, R. L. (1989). TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu Rev Immunol 7, 145-73.

Mosmann, T. R., and Moore, K. W. (1991). The role of IL-10 in crossregulation of TH1 and TH2 responses. Immunol Today 12, A49-53.

Mosmann, T. R., and Sad, S. (1996). The expanding universe of T-cell subsets: Th1, Th2 and more. Immunol Today 17, 138-46.

Muchmore, A. V., and Blaese, R. M. (1977). Immunoregulatory properties of fractions from human pregnancy urine: evidence that human chorionic gonadotropin is not responsible. J Immunol 118, 881-6.

Murphy, G., and Docherty, A. J. (1992). The matrix metalloproteinases and their inhibitors. Am J Respir Cell Mol Biol 7, 120-5.

Nestler, J. E. (1993). Interleukin-1 stimulates the aromatase activity of human placental cytotrophoblasts. Endocrinology 132, 566-70.

Nishino, E., Matsuzaki, N., Masuhiro, K., Kameda, T., Taniguchi, T., Takagi, T., Saji, F., and Tanizawa, O. (1990). Trophoblast-derived interleukin-6 (IL-6) regulates human chorionic gonadotropin release through IL-6 receptor on human trophoblasts. J Clin Endocrinol Metab 71, 436-41.

Norwitz, E. R., Starkey, P. M., Lopez, B. A., and Turnbull, A. C. (1991). Identification by flow cytometry of the prostaglandin-producing cell populations of term human decidua. J Endocrinol 131, 327-34.

O'Hara, R. J. (1995). Antigen-specific suppressor factor: missing pieces in the puzzle. Immunol Res 14, 252-62.

Ogata, Y., Pratta, M. A., Nagase, H., and Arner, E. C. (1992). Matrix metalloproteinase 9 (92-kDa gelatinase/type IV collagenase) is induced in rabbit articular chondrocytes by

cotreatment with interleukin 1 beta and a protein kinase C activator. Exp Cell Res 201, 245-9.

Padykula, H. A., and Driscoll, S. G. (1978). Decidual cell differentiation in the normal early gestational uterus includes lymphoid infiltration. Anat Rec 190, 500-501.

Page, E. W., Villee, C. A., and Villee, D. B. (1981). Human Reproduction. In Essentials of Reproductive and Perinatal Medicine (Philadelphia: WB Saunders).

Parham, P., Barnstable, C. J., and Bodmer, W. F. (1979). Use of a monoclonal antibody (W6/32) in structural studies of HLA-A,B,C, antigens. J Immunol 123, 342-9.

Parhar, R. S., Kennedy, T. G., and Lala, P. K. (1988). Suppression of lymphocyte alloreactivity by early gestational human decidua. I. Characterization of suppressor cells and suppressor molecules. Cell Immunol 116, 392-410.

Parker, D. C. (1993). T cell-dependent B cell activation. Annu Rev Immunol 11, 331-60.

Pattillo, R. A., and Gey, G. O. (1968). The establishment of a cell line of human hormone-synthesizing trophoblastic cells in vitro. Cancer Res 28, 1231-6.

Pattillo, R. A., Ruckert, A. C., Hussa, R. O., Bernstein, R., and E. Delfs (1971). The JAR cell line: continuous human multihormone production and controls. In Vitro 6, 398-405.

Pazmany, L., Mandelboim, O., Vales, G. M., Davis, D. M., Reyburn, H. T., and Strominger, J. L. (1996). Protection from natural killer cell-mediated lysis by HLA-G expression on target cells. Science 274, 792-5.

Peterson, C. M., Schuppert, K., Kelly, P. C., and Pappagianis, D. (1993). Coccidioidomycosis and pregnancy. Obstet Gynecol Surv 48, 149-56.

Pijnenborg, R., Robertson, W. B., Brosens, I., and Dixon, G. (1981). Review article: trophoblast invasion and the establishment of haemochorial placentation in man and laboratory animals. Placenta 2, 71-91.

Pollard, J. W. (1991). Lymphohematopoietic cytokines in the female reproductive tract. Curr Opin Immunol 3, 772-7.

Power, D. A., Catto, G. R., Mason, R. J., MacLeod, A. M., Stewart, G. M., Stewart, K. N., and Shewan, W. G. (1983). The fetus as an allograft: evidence for protective antibodies to HLA-linked paternal antigens. Lancet 2, 701-4.

Pritchard, J. A., Cunningham, F. G., and Mason, R. A. (1976). Coagulation changes in eclampsia: their frequency and pathogenesis. Am J Obstet Gynecol 124, 855-64.

Purcell, D. F., McKenzie, I. F., Lublin, D. M., Johnson, P. M., Atkinson, J. P., Oglesby, T. J., and Deacon, N. J. (1990). The human cell-surface glycoproteins HuLy-m5, membrane co-factor protein (MCP) of the complement system, and trophoblast leucocyte-common (TLX) antigen, are CD46. Immunology 70, 155-61.

Pursley, T. J., Blomquist, I. K., Abraham, J., Andersen, H. F., and Bartley, J. A. (1996). Fluconazole-induced congenital anomalies in three infants. Clin Infect Dis 22, 336-40.

Queenan, J. J., Kao, L. C., Arboleda, C. E., Ulloa, A. A., Golos, T. G., Cines, D. B., and Strauss, J. 3. (1987). Regulation of urokinase-type plasminogen activator production by cultured human cytotrophoblasts. J Biol Chem 262, 10903-6.

Raghupathy, R., Singh, B., and Wegmann, T. G. (1984). Fate of antipaternal H-2 antibodies bound to the placenta in vivo. Transplantation 37, 296-300.

Rammensee, H. G. (1996). Antigen presentation--recent developments. Int Arch Allergy Immunol 110, 299-307.

Ramsey, E. M., Houston, M. L., and Harris, J. W. (1976). Interactions of the trophoblast and maternal tissues in three closely related primate species. Am J Obstet Gynecol 124, 647-52.

Ramsey, E. M. (1982). The Placenta: Human and Animal (New York: Praeger).

Redman, C. W. (1990). Are there immunologically mediated abortions? If so, which mechanisms? Res Immunol 141, 169-75.

Redman, C. W. (1990). Platelets and the beginnings of preeclampsia [editorial; comment]. N Engl J Med 323, 478-80.

Redman, C. W. (1991). Current topic: pre-eclampsia and the placenta. Placenta 12, 301-8.

Reich, R., Thompson, E. W., Iwamoto, Y., Martin, G. R., Deason, J. R., Fuller, G. C., and Miskin, R. (1988). Effects of inhibitors of plasminogen activator, serine proteinases, and collagenase IV on the invasion of basement membranes by metastatic cells. Cancer Res 48, 3307-12.

Reiner, S. L., Zheng, S., Corry, D. B., and Locksley, R. M. (1993). Constructing polycompetitor cDNAs for quantitative PCR. J Immunol Methods 165, 37-46.

Reitamo, S., Remitz, A., Tamai, K., and Uitto, J. (1994). Interleukin-10 modulates type I collagen and matrix metalloprotease gene expression in cultured human skin fibroblasts. J Clin Invest 94, 2489-92.

Remacle-Bonnet, M. M., Rance, R. J., and Depieds, R. C. (1983). Non-specific immunoregulatory factors in the cytosol fraction of human trophoblast. J Reprod Immunol 5, 123-34.

Ries, C., and Petrides, P. E. (1995). Cytokine regulation of matrix metalloproteinase activity and its regulatory dysfunction in disease. Biol Chem Hoppe Seyler 376, 345-55.

Ritson, A., and Bulmer, J. N. (1987). Endometrial granulocytes in human decidua react with a natural-killer (NK) cell marker, NKH1. Immunology 62, 329-31.

Rivas, J. M., and Ullrich, S. E. (1992). Systemic suppression of delayed-type hypersensitivity by supernatants from UV-irradiated keratinocytes. An essential role for keratinocyte-derived IL-10. J Immunol 149, 3865-71.

Roberts, R. M., Cross, J. C., Farin, C. E., Farin, P. W., Kramer, K. K., Francis, H., Librach, C., and Fisher, S. J. (1991). Trophoblast Interferons. In Growth Factors in Reproduction, D. W. Schomberg, ed. (New York: Springer-Verlag), pp. 235-245.

Roberts, J. M., Taylor, C. T., Melling, G. C., Kingsland, C. R., and Johnson, P. M. (1992). Expression of the CD46 antigen, and absence of class I MHC antigen, on the human oocyte and preimplantation blastocyst. Immunology 75, 202-5.

Roberts, J. M., Taylor, R. N., Friedman, S. A., and Goldfien, A. (1993). New developments in pre-eclampsia. In Fetal Medical Review, W. Dunlop, ed. (London: Edward Arnold).

Roby, K. F., Fei, K., Yang, Y., and Hunt, J. S. (1994). Expression of HLA class II-associated peptide transporter and proteasome genes in human placentas and trophoblast cell lines. Immunology 83, 444-8.

Rock, J., Hertig, A.T. (1948). The human conceptus during the first two weeks of gestation. Am. J. Obstet. Gynecol. 55, 6.

Rocklin, R. E., Kitzmiller, J. L., and Kaye, M. D. (1979). Immunobiology of the maternal-fetal relationship. Annu Rev Med 30, 375-404.

Rocklin, R. E., Kitzmiller, J. L., and Garvoy, M. R. (1982). Maternal-fetal relation. II. Further characterization of an immunologic blocking factor that develops during pregnancy. Clin Immunol Immunopathol 22, 305-15.

Romagnani, S. (1991). Human TH1 and TH2 subsets: doubt no more. Immunol Today 12, 256-7.

Roth, I., Corry, D. B., Locksley, R. M., Abrams, J. S., Litton, M. J., and Fisher, S. J. (1996). Human placental cytotrophoblasts produce the immunosuppressive cytokine interleukin 10. J Exp Med 184, 539-48.

Rubinstein, A., Koren, Z., and Murphy, R. A. (1982). Suppression of maternal lymphocyte mitogenic responses by supernatants from short-term placental cell cultures. Am J Reprod Immunol 2, 260-4.

Sabet, L. M., Daya, D., Stead, R., Richmond, H., and Jimeneze, C. L. (1989). Significance and value of immunohistochemical localization of pregnancy specific proteins in feto-maternal tissue throughout pregnancy. Mod Pathol 2, 227-32.

Saito, S., Fukunaga, R., Ichijo, M., and Nagata, S. (1994). Expression of granulocyte colony-stimulating factor and its receptor at the fetomaternal interface in murine and human pregnancy. Growth Factors 10, 135-43.

Saito, S., Kasahara, T., Sakakura, S., Umekage, H., Harada, N., and Ichijo, M. (1994). Detection and localization of interleukin-8 mRNA and protein in human placenta and decidual tissues. J Reprod Immunol 27, 161-72.

Salo, T., Lyons, J. G., Rahemtulla, F., Birkedal, H. H., and Larjava, H. (1991). Transforming growth factor-beta 1 up-regulates type IV collagenase expression in cultured human keratinocytes. J Biol Chem 266, 11436-41.

Sanders, S. K., Giblin, P. A., and Kavathas, P. (1991). Cell-cell adhesion mediated by CD8 and human histocompatibility leukocyte antigen G, a nonclassical major histocompatibility complex class 1 molecule on cytotrophoblasts. J Exp Med 174, 737-40.

Sang, Q. A., and Douglas, D. A. (1996). Computational sequence analysis of matrix metalloproteinases. J Prot Chem 15, 137-159.

Sanyal, M. K., Brami, C. J., Bischof, P., Simmons, E., Barnea, E. R., Dwyer, J. M., and Naftolin, F. (1989). Immunoregulatory activity in supernatants from cultures of normal human trophoblast cells of the first trimester. Am J Obstet Gynecol 161, 446-53.

Schmorl, G. (1893). Pathologisch-Anatomische Untersuchungen über Puerperale Eklampsie. (Leipzig: R.C.W. Vogel).

Schmorl, G. (1905). Über das Schicksal embolisch verschleppter Plazentarzellen. Verh. Dtsch. Pathol. Ges. 8, 39-46.

Schroder, J. (1975). Transplacental passage of blood cells. J Med Genet 12, 230-42.

Shapiro, S. D., Campbell, E. J., Kobayashi, D. K., and Welgus, H. G. (1990). Immune modulation of metalloproteinase production in human macrophages. Selective pretranslational suppression of interstitial collagenase and stromelysin biosynthesis by interferon-gamma. J Clin Invest 86, 1204-10.

Sheppard, B. L., and Bonnar, J. (1974). The ultrastructure of the arterial supply of the human placenta in early and late pregnancy. J Obstet Gynaecol Br Commonw 81, 497-511.

Sherman, L. A., and Chattopadhyay, S. (1993). The molecular basis of allorecognition. Annu Rev Immunol 11, 385-402.

Shimizu, Y., and DeMars, R. (1989). Demonstration by class I gene transfer that reduced susceptibility of human cells to natural killer cell-mediated lysis is inversely correlated with HLA class I antigen expression. Eur J Immunol 19, 447-51.

Shukla, H., Swaroop, A., Srivastava, R., and Weissman, S. M. (1990). The mRNA of a human class I gene HLA G/HLA 6.0 exhibits a restricted pattern of expression. Nucleic Acids Res 18, 2189.

Siiteri, P. K., Febres, F., Clemens, L. E., Chang, R. J., Gondos, B., and Stites, D. (1977). Progesterone and maintenance of pregnancy: is progesterone nature's immunosuppressant? Ann N Y Acad Sci 286, 384-97.

Siiteri, P. K., and Stites, D. P. (1982). Immunologic and endocrine interrelationships in pregnancy. Biol Reprod 26, 1-14.

Silbiger, S. M., Jacobsen, V. L., Cupples, R. L., and Koski, R. A. (1994). Cloning of cDNAs encoding human TIMP-3, a novel member of the tissue inhibitor of metalloproteinase family. Gene 141, 293-7.

Silver, R. K., Turbov, J. M., Beaird, J. A., and Golbus, J. (1990). Soluble factors produced by isolated first-trimester chorionic villi directly inhibit proliferation of T cells. Am J Obstet Gynecol, 1914-9.

Sionov, R. V., Yagel, S., Har, N. R., and Gallily, R. (1993). Trophoblasts protect the inner cell mass from macrophage destruction. Biol Reprod 49, 588-95.

Sipes, S. L., Medaglia, M. V., Stabley, D. L., DeBruyn, C. S., Alden, M. S., Catenacci, V., and Landel, C. P. (1996). A new major histocompatibility complex class I b gene expressed in the mouse blastocyst and placenta. Immunogenetics 45, 108-20.

Sleckman, B. P., Gorman, J. R., and Alt, F. W. (1996). Accessibility control of antigen-receptor variable-region gene assembly: role of cis-acting elements. Annu Rev Immunol 14, 459-81.

Sonnenberg, A., Linders, C. J., Daams, J. H., and Kennel, S. J. (1990). The alpha 6 beta 1 (VLA-6) and alpha 6 beta 4 protein complexes: tissue distribution and biochemical properties. J Cell Sci, 207-17.

Spector, T. D. (1990). Rheumatoid arthritis. Rheum Dis Clin North Am 16, 513-37.

Spornitz, U. M. (1992). The functional morphology of the human endometrium and decidua. Adv Anat Embryol Cell Biol 124, 1-99.

Starkey, P. M., Sargent, I. L., and Redman, C. W. (1988). Cell populations in human early pregnancy decidua: characterization and isolation of large granular lymphocytes by flow cytometry. Immunology 65, 129-34.

Stein, K. E. (1992). Thymus-independent and thymus-dependent responses to polysaccharide antigens. J Infect Dis, S49-52.

Stephanou, A., and Handwerger, S. (1994). Interleukin-6 stimulates placental lactogen expression by human trophoblast cells. Endocrinology 135, 719-23.

Sthoeger, Z. M., Chiorazzi, N., and Lahita, R. G. (1988). Regulation of the immune response by sex hormones. I. In vitro effects of estradiol and testosterone on pokeweed mitogen-induced human B cell differentiation. J Immunol 141, 91-8.

Story, C. M., Mikulska, J. E., Simister, N. E. (1994). A major histocompatibility complex class I-like Fc receptor cloned from human placenta: possible role in transfer of immunoglobulin G from mother to fetus. J Exp Med 180(6):2377-81.

Strauss, J. 3., Martinez, F., and Kiriakidou, M. (1996). Placental steroid hormone synthesis: unique features and unanswered questions. Biol Reprod 54, 303-11.

Sunderland, C. A., Redman, C. W., and Stirrat, G. M. (1981). HLA A, B, C antigens are expressed on nonvillous trophoblast of the early human placenta. J Immunol 127, 2614-5.

Suwa, S., and Friesen, H. (1969). Biosynthesis of human placental proteins and human placental lactogen (HPL) in vitro. II. Dynamic studies of normal term placentas. Endocrinology 85, 1037-45.

Tachi, C., and Tachi, S. (1989). Role of macrophages in the maternal recognition of pregnancy. J Reprod Fertil Suppl 37, 63-8.

Takeshita, S., Gage, J. R., Kishimoto, T., Vredevoe, D. L., and Martinez, M. O. (1996). Differential regulation of IL-6 gene transcription and expression by IL-4 and IL-10 in human monocytic cell lines. J Immunol 156, 2591-8.

Takimoto, E., Ishida, J., Sugiyama, F., Horiguchi, H., Murakami, K., and Fukamizu, A. (1996). Hypertension induced in pregnant mice by placental renin and maternal angiotensinogen [see comments]. Science 274, 995-8.

Tallon, D. F., Corcoran, D. J., O'Dwyer, E. M., and Greally, J. F. (1984). Circulating lymphocyte subpopulations in pregnancy: a longitudinal study. J Immunol 132, 1784-7.

Talmage, D. W. (1986). The acceptance and rejection of immunological concepts. Annu Rev Immunol 4, 1-11.

Tao, Y. X., and Cao, Y. Q. (1993). Modulation of interferon secretion by concanavalin A and interleukin-2 in first trimester placental explants in vitro. J Reprod Immunol 24, 201-12.

Teasdale, F., and Jean-Jacques, G. (1985). Morphometric evaluation of the microvillous surface enlargement factor in the human placenta from mid-gestation to term. Placenta 6, 375-81.

Tedesco, F., Narchi, G., Radillo, O., Meri, S., Ferrone, S., and Betterle, C. (1993). Susceptibility of human trophoblast to killing by human complement and the role of the complement regulatory proteins. J Immunol 151, 1562-70.

Tomlinson, S. (1993). Complement defense mechanisms. Curr Opin Immunol 5, 83-9.

Topalovski, M., Yang, S. S., and Boonpasat, Y. (1993). Listeriosis of the placenta: clinicopathologic study of seven cases. Am J Obstet Gynecol 169, 616-20.

Trinchieri, G. (1995). Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. Annu Rev Immunol 13, 251-76.

Truman, P., and Ford, H. C. (1984). The brush border of the human term placenta. Biochim Biophys Acta 779, 139-60.

Unemori, E. N., Hibbs, M. S., and Amento, E. P. (1991). Constitutive expression of a 92-kD gelatinase (type V collagenase) by rheumatoid synovial fibroblasts and its induction in normal human fibroblasts by inflammatory cytokines. J Clin Invest 88, 1656-62.

van der Ven, K., and Ober, C. (1994). HLA-G polymorphisms in African Americans. J Immunol 153, 5628-33.

van Oers, N. S., Cohen, B. L., and Murgita, R. A. (1989). Isolation and characterization of a distinct immunoregulatory isoform of alpha-fetoprotein produced by the normal fetus. J Exp Med 170, 811-25.

van Rood, J. J., Eernisse, J. G., and van Leeuwen, A. (1958). Leucocyte antibodies in sera from pregnant women. Nature 181, 1735-1736.

Vanderpuye, O. A., and Smith, C. H. (1987). Proteins of the apical and basal plasma membranes of the human placental syncytiotrophoblast: immunochemical and electrophoretic studies. Placenta 8, 591-608.

Varner, M. W. (1991). Autoimmune disorders and pregnancy. Semin Perinatol 15, 238-50.

Vassali, P., Morris, R., and McCluskey, R. I. (1963). The pathogenic role in fibrin deposition in the glomerular lesions of toxemia of pregnancy. J. Exp. Med. 118, 467-477.

Vince, G., Shorter, S., Starkey, P., Humphreys, J., Clover, L., Wilkins, T., Sargent, I., and Redman, C. (1992). Localization of tumour necrosis factor production in cells at the materno/fetal interface in human pregnancy. Clin Exp Immunol 88, 174-80.

Vince, G. S., and Johnson, P. M. (1995). Materno-fetal immunobiology in normal pregnancy and its possible failure in recurrent spontaneous abortion? Hum Reprod 2, 107-13.

Wahl, S. M., Allen, J. B., Weeks, B. S., Wong, H. L., and Klotman, P. E. (1993). Transforming growth factor beta enhances integrin expression and type IV collagenase secretion in human monocytes. Proc Natl Acad Sci U S A 90, 4577-81.

Watanabe, H., Nakanishi, I., Yamashita, K., Hayakawa, T., and Okada, Y. (1993). Matrix metalloproteinase-9 (92 kDa gelatinase/type IV collagenase) from U937 monoblastoid cells: correlation with cellular invasion. J Cell Sci, 991-9.

Weber, N. R., Riley, J. K., Greenlund, A. C., Moore, K. W., Darnell, J. E., and Schreiber, R. D. (1996). Stat3 recruitment by two distinct ligand-induced, tyrosine-phosphorylated docking sites in the interleukin-10 receptor intracellular domain. J Biol Chem 271, 27954-61.

Wecker, H., and Auchincloss, H. J. (1992). Cellular mechanisms of rejection. Curr Opin Immunol 4, 561-6.

Wegmann, T. G., Mosmann, T. R., Carlson, G. A., Olijnyk, O., and Singh, B. (1979). The ability of the murine placenta to absorb monoclonal anti-fetal H-2K antibody from the maternal circulation. J Immunol 123, 1020-3.

Wegmann, T. G., Singh, B., and Carlson, G. A. (1979). Allogeneic placenta is a paternal strain antigen immunoabsorbent. J Immunol 122, 270-4.

Wegmann, T. G. (1980). Why didn't your mother reject you? Can Med Assoc J 123, 991-3.

Wegmann, T. G., Lin, H., Guilbert, L., and Mosmann, T. R. (1993). Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a TH2 phenomenon? Immunol Today 14, 353-6.

Wehinger, J., Gouilleux, F., Groner, B., Finke, J., Mertelsmann, R., and Weber, N. R. (1996). IL-10 induces DNA binding activity of three STAT proteins (Stat1, Stat3, and Stat5) and their distinct combinatorial assembly in the promoters of selected genes. Febs Lett 394, 365-70.

Weinberg, P. C., Cameron, I. L., Parmley, T., Jeter, J. R., and Pauerstein, C. J. (1970). Gestational age and placental cellular replication. Obstet Gynecol 36, 692-6.

Weinberg, E. D. (1984). Pregnancy-associated depression of cell-mediated immunity. Rev Infect Dis 6, 814-31.

Weingarten, H., Martin, R., and Feder, J. (1985). Synthetic substrates of vertebrate collagenase. Biochemistry 24, 6730-4.

Werb, Z., Mainardi, C. L., Vater, C. A., and Harris, E. J. (1977). Endogenous activiation of latent collagenase by rheumatoid synovial cells. Evidence for a role of plasminogen activator. N Engl J Med 296, 1017-23.

Westphal, J. R., Willems, H. W., Ruiter, D. J., and De, W. R. (1993). Involvement of LFA-1/ICAM and CD2/LFA-3 in human endothelial cell accessory function. Behring Inst Mitt, 51-62.

Witebsky, E., and Reich, H. (1932). Zur gruppenspezi fischen differenzierung der placentarorgane. Klin. Wochenschr. 11, 1960-1961.

Woods, J. J., Plessinger, M. A., and Clark, K. E. (1987). Effect of cocaine on uterine blood flow and fetal oxygenation. JAMA 257, 957-61.

Wynn, R. M. (1964). Comparative morphogenesis and vascular relationships of the villous hemochorial placenta. Am. J. Obstet. Gynecol. 90, 758-768.

Wynn, R. M., and Davies, J. (1965). Comparative electron microscopy of the hemochorial placenta. Am. J. Obstet. Gynecol. 91, 533-549.

Yagel, S., Palti, Z., and Gallily, R. (1988). Prostaglandin E2-mediated suppression of human maternal lymphocyte alloreactivity by first-trimester fetal macrophages. Obstet Gynecol 72, 648-54.

Yelavarthi, K. K., Fishback, J. L., and Hunt, J. S. (1991). Analysis of HLA-G mRNA in human placental and extraplacental membrane cells by in situ hybridization. J Immunol 146, 2847-54.

York, I. A., and Rock, K. L. (1996). Antigen processing and presentation by the class I major histocompatibility complex. Annu Rev Immunol 14, 369-96.

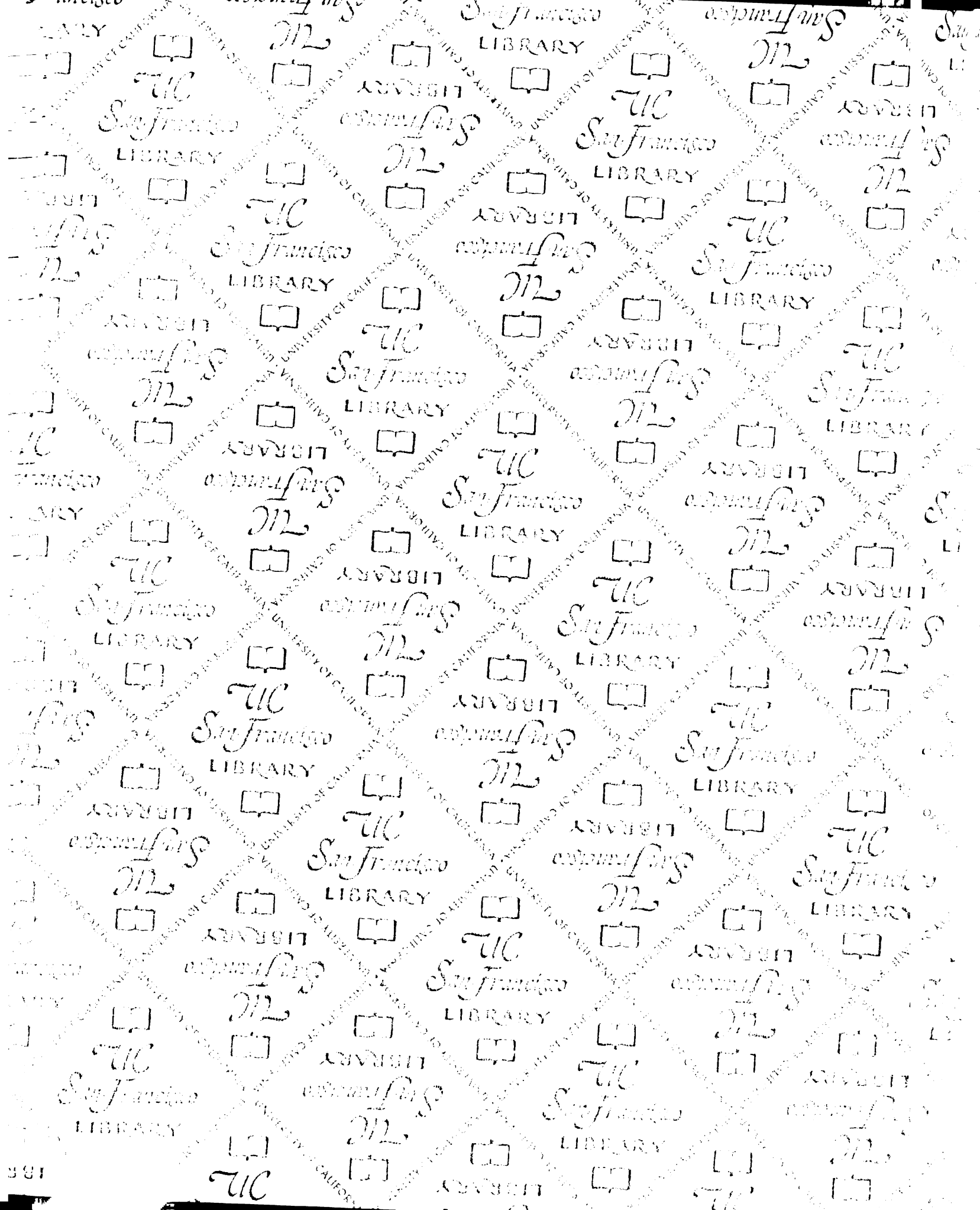
Yow, M. D. (1989). Congenital cytomegalovirus disease: a NOW problem [see comments]. J Infect Dis 159, 163-7.

Zhou, Y., Damsky, C. H., Chiu, K., Roberts, J. M., and Fisher, S. J. (1993). Preeclampsia is associated with abnormal expression of adhesion molecules by invasive cytotrophoblasts. J Clin Invest 91, 950-60.

Zhou, Y., Damsky, C. H., and Fisher, S. J. (1997). Preeclampsia is associated with failure of human cytotrophoblasts to mimic a vascular adhesion phenotype: one cause of defective endovascular invasion in this syndrome? Journal of Clinical Investigation.

Zhou, Y., Fisher, S. J., Janatpour, M. J., Genbacev, O., Dejana, E., Wheelock, M., and Damsky, C. H. (1997). Human cytotrophoblasts adopt a vascular phenotype as they differentiate: a strategy for successful endovascular invasion? Journal of Clinical Investigation (In Press).

Zlotnik, A. and Moore, T. A. (1995). Cytokine production and requirements during T-cell development. Curr Opin Immunol, 7(2):206-13.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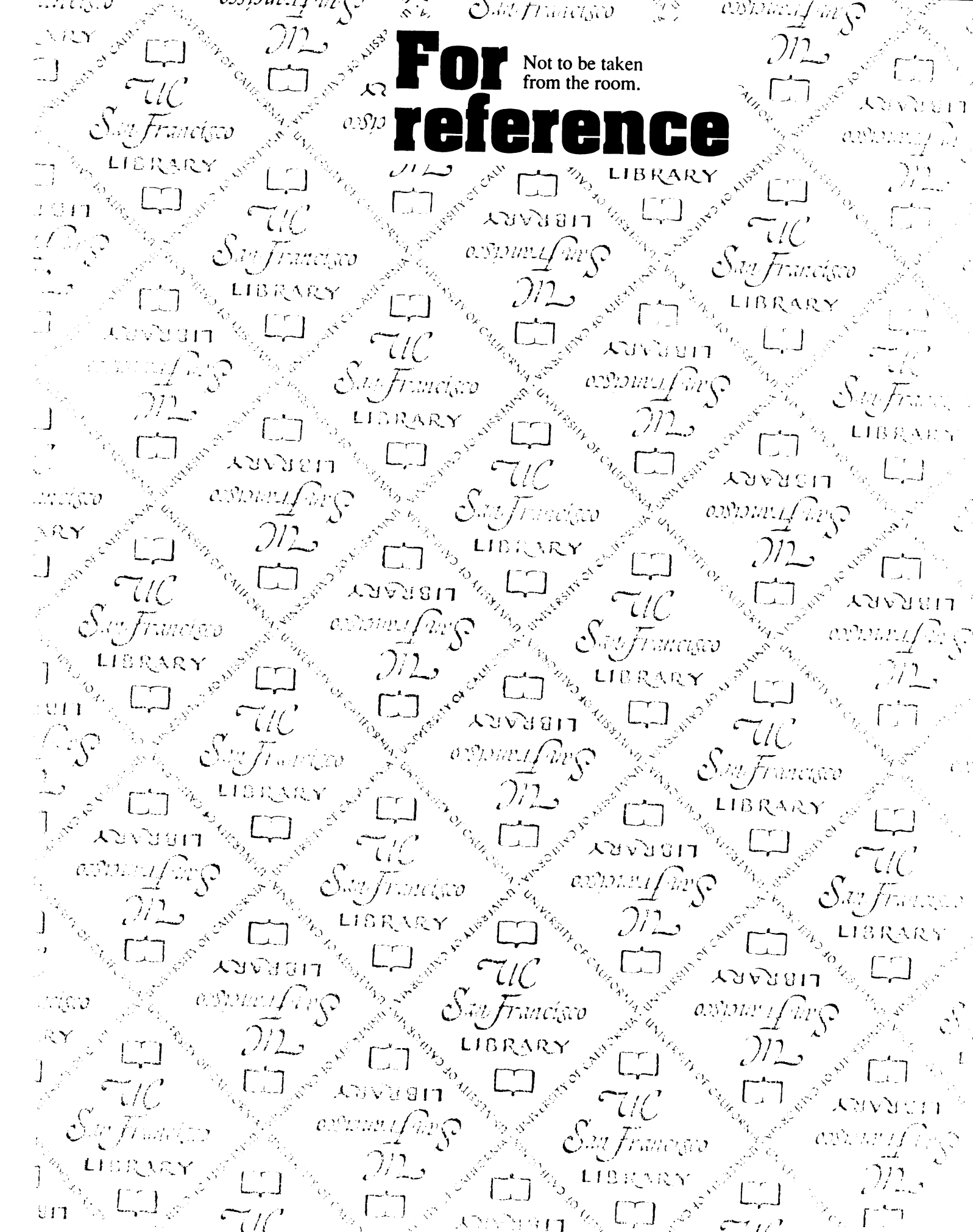
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