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Immunologic Evaluation of Ectopic Gestation

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IMMUNOLOGIC EVALUATION OF ECTOPIC GESTATION

by

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ABSTRACT

IMMUNOLOGIC EVALUATION OF
ECTOPIC GESTATION

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Ectopic gestation is an error in the implantation site of the blastocyst. Extrauterine implantation presents a unique environment since the protective features of the uterus are absent. This study is an investigation into the immunoregulatory differences that may develop as a result of a change in the feto-maternal junction.

The effects of female plasma on one-way mixed lymphocyte cultures and mitogen-stimulated cultures, anti-paternal lymphocytotoxicity, and lymphocyte phenotypes were determined on normal, ectopic and non-pregnant females. The results indicated that pregnant female plasma enhanced the response of autologous lymphocytes to spouse and male control lymphocytes. Enhancement was not seen in the mixed lymphocyte cultures from non-pregnant, ectopic females, or unrelated males. Lymphocytes from ectopic females were not responsive to plasma from normal pregnant females. There was no differences in the mitogen cultures and leukocyte phenotypes among the three pregnancy conditions. These results suggest the presence of some factor(s) in the plasma of pregnant females capable of enhancing autologous lymphocytes. Such factor(s) and the enhancement of lymphocyte responses seem to be lacking in cultures from ectopic pregnant and non-pregnant females.

To my father for his principles, values, wisdom, and love of learning

To my mother for her ambition, leadership, kindness, and piety

To my wife for her love, counsel, forbearance, and assistance

To my sister for her encouragement, sincerity, and endurance

To all of you for your love, sacrifice, and support

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CHAPTER ONE

Background of the Problem

Introduction

Termed "an unmitigated disaster of reproduction", (Cunningham, MacDonald, & Gant, 1989), ectopic pregnancy has been on the rise since 1970 - the year when the National Center for Health Statistics (NCHS) started monitoring the condition. Ectopic pregnancy is the clinical condition in which the blastocyst implants in sites other than its normal anatomical site - the lining of the uterine cavity. Sites of ectopic pregnancy are classified as tubal, ovarian, cervical or abdominal. In the fallopian tube, the ampulla is the most common area for the occurrence of ectopic pregnancy, followed by the isthmus. Interstitial pregnancy is the most uncommon site for implantation, occurring in about 3% of tubal pregnancies. Other forms of ectopic pregnancy (Figure 1) involve the tubo-ovarian region, the ovaries, and the tubo-abdominal area (Cunningham et al., 1989).

Ectopic pregnancy is becoming a public health burden in the United States and other countries all over the world. High rates of morbidity are associated with ectopic pregnancy including a significant decline in the reproductive capabilities of women, and an increase in the risk of another ectopic gestation (Trussell, Hatcher, Cates, Stewart, & Kost, 1990).

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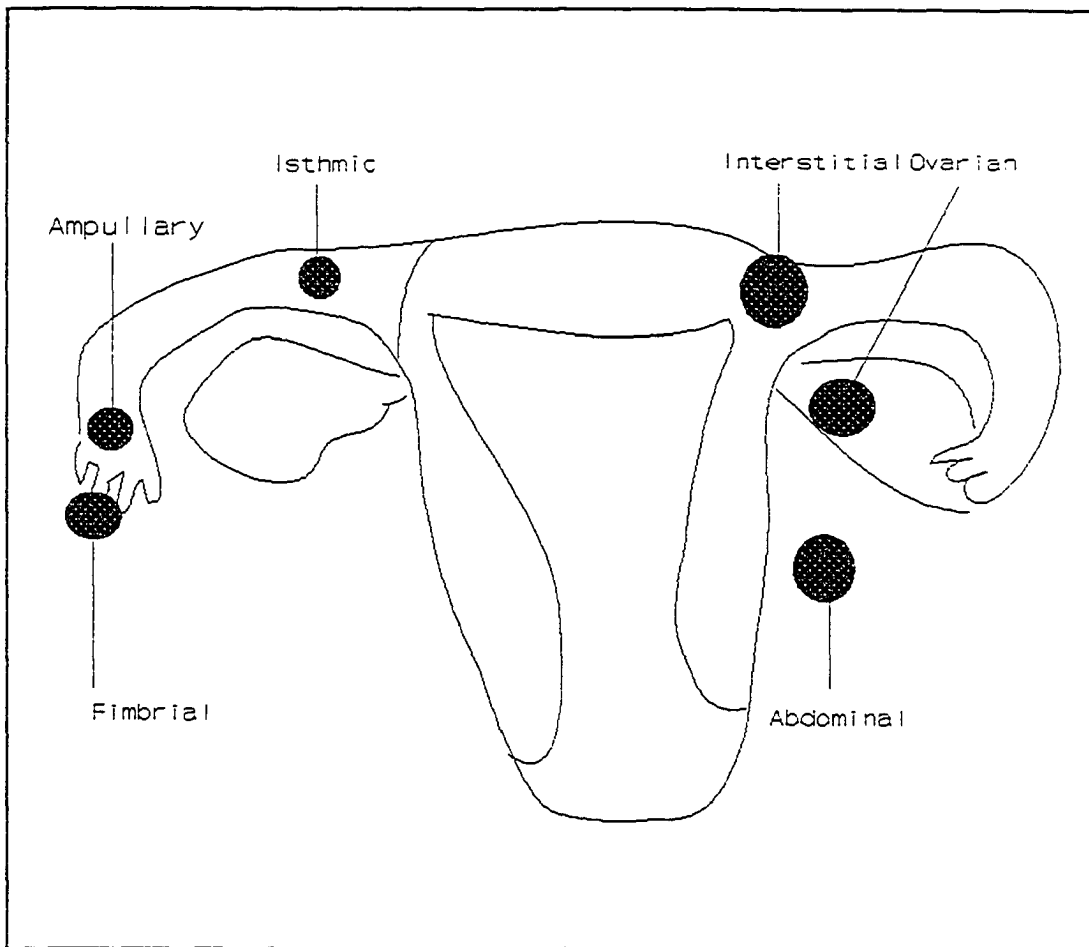
Figure 1. Various sites of ectopic gestation

In addition to the health risks associated with this condition, the economic repercussions are by no means less dramatic. Using data from 1982-1989, Washington and Katz (1991; 1993) estimate the indirect and direct costs of ectopic pregnancy to be $1.1 billion in 1990 or $9,482 per case. Moreover, 35% of the direct costs were paid out of public sources for conditions in women 19 years of age or younger, while private insurance paid the greater share in women age 20 or older. The greatest share (67%) of indirect costs came from lost wages. Retrospective analysis of ectopic pregnancy admissions at one hospital between the years 1970 - 1974 and 1980 - 1984 show an increase in the incidence of ectopic pregnancy among young, minority, unmarried, poor females with no insurance. The increase in incidence among younger women is primarily due to an increase in conditions such as sexually transmitted disease that predispose them to ectopic pregnancy (Krantz, Gray, Damewood, & Wallach, 1990).

Epidemiology and incidence

Ectopic gestation reached an alarming rate (16.8 per 1000 reported pregnancies) in 1987. After a slight decline in 1988, the rate of ectopic pregnancy rose back to 16.1 in 1989 and approached the rate in 1987 (Nederlof, Lawson, Saftlas, Atrash, & Finch, 1990). Figures 2 and 3 illustrate the rise in reported cases and rates between the years 1970 and

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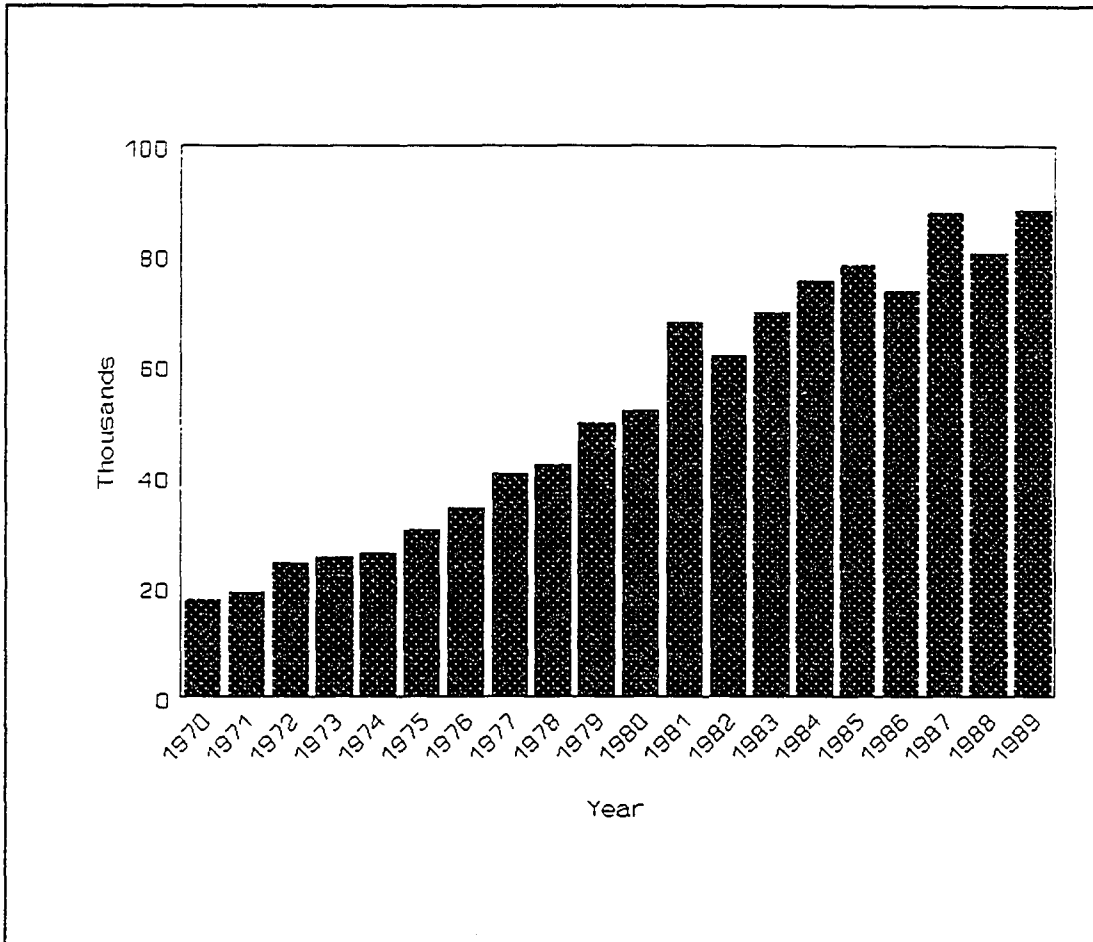
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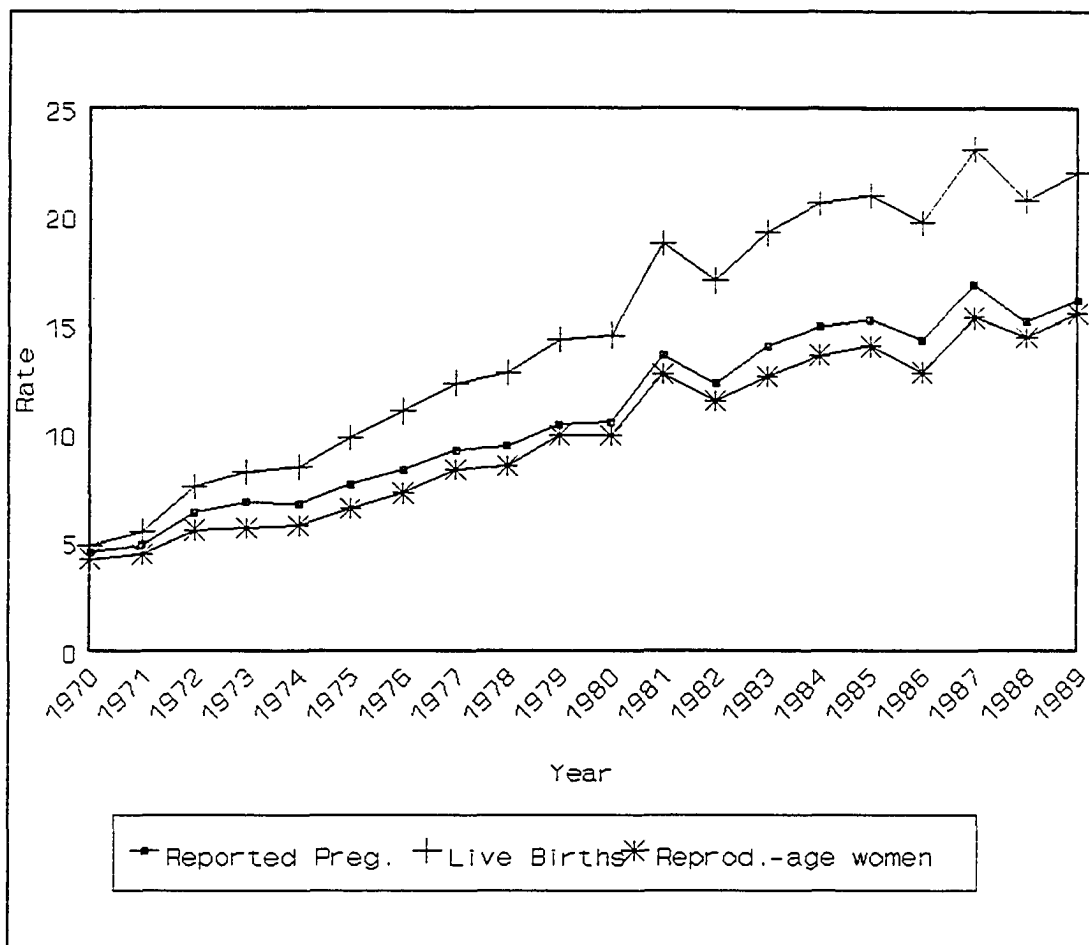
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1989, respectively. Ectopic gestation rates, however, must be analyzed with great care. The rates do not include federal hospitals, and many ectopic pregnancies go undiagnosed due to spontaneous resolution. Inconsistencies in reporting result in an imprecise assessment of the prevalence of ectopic pregnancy. Using all women of reproductive age (15-44 years old) may give the best assessment for the incidence and prevalence of this condition (Doyle, DeCherney, & Diamond, 1991). Ectopic pregnancies represent about 1.5% of all pregnancies in the United States and 0.5% of all pregnancies in women ages 15 - 24 years (Ammerman, Shafer, & Snyder, 1990). Currently, complications of ectopic pregnancy are the principle cause of death during the first trimester and the second leading cause of death associated with pregnancy. Death certificates filed at the NCHS show that death due to ectopic pregnancy is three times greater among African-American and minority women (Figure 4) than among their white counterparts (Centers for Disease Control and Prevention, 1992). Moran, Aral, Jenkins, Peterman, and Alexander (1989) demonstrate that the high rate of sexually transmitted diseases among minority populations predispose them to an increased risk of ectopic pregnancy due to reproductive organ damage. Analysis of data collected in the 1970's and early 1980's reveal that women who died from ectopic pregnancy experienced pain prior to the rupture of the tube. Thirty percent died at

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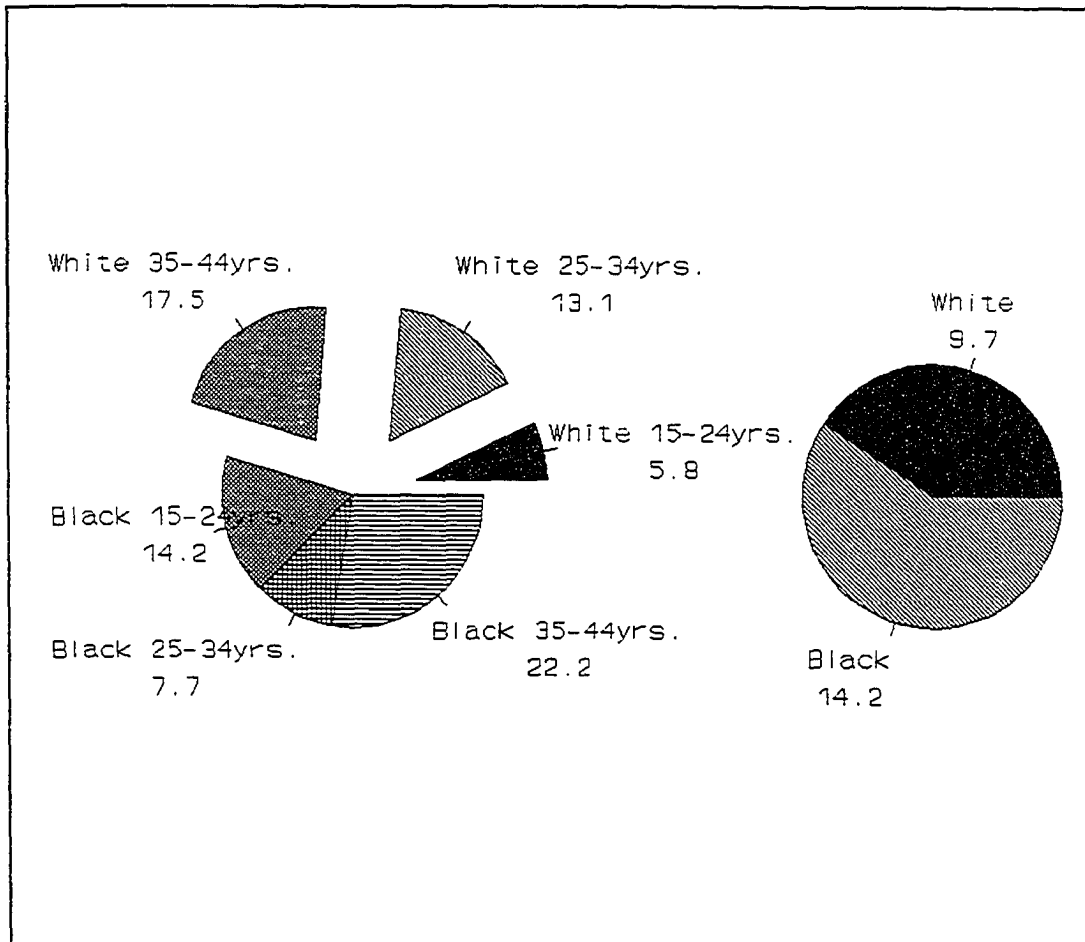
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home and hemorrhage was the leading cause of death (85% of all deaths, 70% of which did not have surgery). Seventy-seven percent of patients who died from ectopic pregnancy were seen by a physician and 49% were misdiagnosed (Dorfman, Grimes, Cates, Binkin, Kafrissen, & O'Reilly, 1984; Atrash, Hughes, & Hogue, 1986).

The increased rates of ectopic gestation is not limited to the United States but has been documented in many other western regions such as Scandinavia, the United Kingdom, and Eastern Europe (Makinen, Erkkola, & Laippala, 1989). Underscoring the high prevalence of ectopic pregnancy in the United States, Stock (1990) report that between the years 1970 and 1980 the percent maternal mortality from ectopic pregnancy was 6.7% in Zambia, 9.8% in Japan, 10% in Jamaica, 14% in Hong Kong as compared with 20% in South Carolina.

Etiology

Cunningham et al. (1989) divides the causes of ectopic pregnancy into two general categories, mechanical and functional. Included under mechanical causes are disorders such as salpingitis, peritubal adhesions, developmental abnormalities of the fallopian tube, previous ectopic pregnancy, past tubal surgeries, abortions and fallopian tube tumors. All these conditions hamper or block the free movement of the ovum or the

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blastocyst through the tube. Functional elements include altered tubal motility due to changes in hormonal levels and less critical conditions such as menstrual reflux and the migration patterns of the ovum. Conditions such as pelvic inflammatory disease and other sexually transmitted diseases are high-risk factors for ectopic gestation (Krantz, Gray, Damewood, & Wallach, 1990). Consequently, an increase in human behaviors that facilitate these clinical conditions will invariably cause a proportional increase in the rate of ectopic pregnancy.

Scarring of the fimbria and tube account for most cases of ectopic pregnancy. This may be caused by infection, inflammation, surgery, and diethylstilbestrol. Chlamydia and gonorrhea are the most common infections that result in distortion, deciliation, and destruction of the fimbria and tubal epithelium (Doyle, DeCherney, & Diamond, 1990). In Finland, a study of 1,017 patients from 1966 to 1985 show that pelvic operations, previous ectopic pregnancies, pelvic inflammatory diseases and the use of intra-uterine devices (IUD) are the most common etiologies for ectopic gestation (Makinen et al., 1989). An Italian case-control study of the risk factors for ectopic pregnancy (Parazzini, Tozzi, Ferraroni, Bocciolone, Vecchia, & Fedele, 1992) confirm previous studies that implicate infertility, history of PID, and abdominal surgery to be among the main risk factors for ectopic pregnancy.

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Infertility drugs and procedures, and therapeutic abortions are other medical procedures that place female recipients in the high-risk group (Damjanov, 1993; Cunningham et al., 1989; Suanders, Lancaster, & Pedisich, 1992). A study of the risk of ectopic gestation and its association with various methods of contraception indicate that intrauterine devices are associated with the highest incidence of ectopic pregnancy. However, this study also show the incidence of ectopic pregnancy to be highest in women who do not use contraception (Franks, Beral, Cates, & Houge, 1990). Even though the risk of ectopic pregnancy and contraceptive use is an ongoing debate between researchers, there is consensus that women who do not use contraception have the highest incidence of ectopic gestation while IUD users have the highest incidence among women using contraceptives (Trussell, Hatcher, Cates, Stewart, & Kostet, 1990; Franks, Beral, Cates, & Hogueet 1990; Sivin, 1991a). Their results are not surprising since IUD's only prevent intrauterine pregnancy and do not protect female users from having an extrauterine pregnancy. In addition, intra-uterine devices do not prevent sexually transmitted diseases and other conditions that result in a higher risk of ectopic gestation. Analysis of randomized trials of various kinds of intrauterine devices show that the risk of ectopic pregnancy depends on the drug and dose of medicated IUD's and on the age of the user (Sivin, 1991b).

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Pathology and diagnosis

With all the information present regarding the physiology of a successful pregnancy, the implantation stage of this process is not comprehensively understood. The maternal and embryonic mechanisms of both normal and abnormal pregnancies require further investigation (Damjanov, 1993; Glasser, Munir, & Soares, 1987; Alpin, 1991).

Ectopic gestation is an error in implantation with the most common anatomical site being the ampulla and isthmus of the fallopian tube. The blastocyst adheres to and penetrates through the tubal mucosa reaching the muscularis of the tube. This action triggers maternal vascularization surrounding the implantation site. The mucosa of the fallopian tube does not undergo a well defined decidual reaction (Cunningham et al., 1989).

The uterus undergoes changes during ectopic pregnancy that mimic a normal pregnancy. Despite the implantation site being distant from the uterine cavity, the uterus under the influence of estrogen and progesterone is converted to decidua. These uterine changes persist as long as the developing trophoblast continues to produce and induce pregnancy hormones (Damjanov, 1993).

Pelvic examinations of subjects with ectopic pregnancy present similar signs of normal early pregnancy such as softening of the cervix and slight enlargement of the uterus. The most critical finding before rupture is a

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sausage-like mass in the adnexal region. The cul-de-sac may be enlarged and signs of peritoneal irritation may be present if there has been bleeding within the peritoneal cavity. Abdominal and pelvic pain are commonly seen in ectopic pregnancy. Vaginal bleeding is seen as a result of the sloughing of the decidua indicating a failure in normal placentation. Other pelvic findings include tenderness in the adnexal region, especially upon elevating or moving the cervix (Cunningham et al., 1989; Damjanov, 1993).

The early diagnosis of ectopic pregnancy has been greatly improved by technological refinements in the pregnancy test. Enzyme immunoassay of the beta subunit of human chorionic gonadotropin (β-HCG) is the most useful laboratory test. The absence of β-HCG virtually rules out viable trophoblast either in normal or abnormal locations. In normal pregnancy β-HCG is expected to double every 48-72 hours. This doubling is not seen in the majority (67%) of ectopic pregnancies (Kadar, DeVore, & Romero, 1981; Kadar, Caldwell, & Romero, 1981; Kadar, & Romero, 1987). Cartwright (1991) reports that β-HCG remained below 200 mIU/mL in 75% of ectopic pregnancies while it may reach 153,000 mIU/ml or more in normal pregnancies. Progesterone has been recently used in the diagnosis of ectopic pregnancy. Progesterone increases in normal pregnancy. A decrease in progesterone levels below 5ng/ml may indicate ectopic gestation (Stovall, Ling, Carson, & Buster, 1990).

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Ultrasound is a helpful tool in the diagnosis of ectopic pregnancy. A normally situated gestational sac in the uterine cavity excludes the existence of an ectopic pregnancy except in the very rare case where it may coexist with a normal pregnancy. Therefore, an increase in β-HCG level and no ultrasonic evidence of intrauterine pregnancy 6 weeks or more after the onset of the last menstrual period is highly diagnostic of ectopic pregnancy (Cunningham et al., 1989). The concept of an HCG discriminatory zone is used with ultrasound to diagnose patients when the HCG level is known. The discriminatory zone for abdominal ultrasound refers to β-HCG levels, 6000-6500 mIU/ml, at which the ultrasound should show an intrauterine gestational sac in a normal pregnancy. The β-HCG level for vaginal probe ultrasound is 1500-2000 mIU/ml. Failure to visualize a normal intrauterine implantation is highly suggestive of ectopic gestation (Kadar et al., 1981).

Laparoscopy is a powerful technique that revolutionized the diagnosis and management of ectopic pregnancy. Laparoscopy has a great advantage in that it may be initiated to rule out ectopic pregnancy through an easy and accurate procedure. If ectopic pregnancy is seen then an operation to remove the fetus can be immediately performed.

Other important laboratory tests are hemoglobin and hematocrit. White blood cell count may be normal but in many patients an increase up to 30,000/μl has been documented (Cunningham et al., 1989).

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Ectopic gestation remains a public health concern due to its high rates of morbidity and its significant mortality rates. The most common etiological factors have been identified; hence, hormonal, ultrasonographic and laparoscopic studies may be employed to rule out ectopic gestation. However, little is known regarding the contributions of embryonic factors to extrauterine implantation and the role they play in the onset of ectopic gestation. Further studies comparing the interaction of the embryo with the tubal and with the normal intrauterine environment may provide further information about placentation in both normal and ectopic pregnancy.

Theoretical Framework

Some of the most basic natural phenomena remain enigmatic despite the innovations in medical and biomedical research. None of these is more fascinating than the survival mechanism of the fetus, a natural semi-allogeneic transplant in the recipient mother.

At implantation, fetal tissue in the form of trophoblast becomes intimately associated with the circulatory system of the mother. Despite fetal expression of paternal antigens that are foreign to the immune system of the mother, maternal immunity remains tolerant for nine months.

What, then, are the pathway(s) that lead to maternal immunologic tolerance towards the fetus? Tolerance is defined as the state of lack of

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immunity (Roitt, Brostoff, & Male, 1989). It is achieved through a number of different pathways that include both T and B lymphocytes. The ultimate result is a state of no immunity, or tolerance, to recognized self antigens. In most transplants, allogeneic or semi-allogeneic tissue evoke an adverse immune response that leads to rejection of the transplanted tissue. Fetal tissue, however, escapes rejection by the maternal immune system. Fetal survival seems to challenge current tolerance theory; namely, the mother is tolerant to non-self antigens during pregnancy. Part of this tolerance is attributed to the "invisibility" of trophoblastic antigens at the maternal interface. The inability of the mother to recognize fetal antigens was shown to be due to the lack of traditional HLA class I and II antigens on the cell surfaces of fetal tissue in direct contact with maternal circulation (Sunderland, Nauem, Mason, Redmen, & Stirratet, 1981).

Even though the traditional HLA antigens are missing, the trophoblast is not immunologically neutral (Sutton, Mason & Redman, 1983). Thus, the maternal immune system must be contributing to the state of tolerance towards the fetus. Since the blastocyst normally implants in the uterus, it is possible that the intrauterine environment provides a unique interface that is responsible for maternal immunomodification. Ectopic gestation is a naturally-occurring experiment in which the implantation site in altered. The objective of the experimental design is to investigate changes in maternal

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immune responses relative to changing the implantation site. Ectopic trophoblast invades areas with a relatively thin muscularis layer, compared to the uterus, and with incomplete decidualization at the implantation site. This change in the feto-maternal interface may provide additional data on the contribution of the normal intrauterine environment to fetal success.

Immunologic assessment of females with unexplained recurrent spontaneous abortion show that increased sharing of HLA alleles between mating couples predispose the females to miscarriage. Normal pregnant females do not share as many HLA alleles with their mates (Gerencer, Drazancic, Kuvacic, Tomaskovic, & Kastelan, 1979). In addition, plasma from normal pregnancy contains blocking antibodies that inhibit mitogen and mixed lymphocyte cultures. These antibodies are responsible for protecting the fetus from adverse maternal immune reactions (Jenkins & Hancock, 1972; Harva & Jouppila, 1977; Jha, Talwar, & Hingorani, 1974). The presence of anti-paternal lymphocytotoxic antibodies is another immunologic feature of maternal plasma that correlates with successful implantation (Taylor & Faulk, 1981). Finally, studies regarding leukocyte populations show that normal T-cell populations are present at the intrauterine but not at tubal implantation sites (Earl, Lunny , & Bulmer, 1987).

The aforementioned findings regarding normal pregnancy were the focus of the immunologic evaluation of ectopic gestation, and guided the

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selection of the experimental design of this study. Mixed lymphocyte and mitogen cultures were used to study HLA diversity among couples and the effects of immunoregulatory factors in blood plasma. Anti-paternal lymphocytotoxicity assay was used to detect the presence of cytotoxic antibodies in maternal serum of the participating females, and leukocyte phenotyping were compared among the three pregnancy conditions.

The study was designed to explain how cellular and humoral responses are modified in ectopic pregnancy as compared to normal pregnancy. In doing so, the study may provide data explaining the relationship between the feto-maternal interface and maternal immune response.

Operational Definitions

Immune function in the study refers to immune responses measured by the mixed lymphocyte response, lymphocytotoxicity, and leukocyte phenotypes.

Ectopic gestation (pregnancy) is the erroneous implantation of the blastocyst in an anatomical site other than the lining of the uterus (Cunningham et al., 1989, p. 511). Ectopic pregnancy in this research study was confirmed through laparoscopic surgery.

One-way mixed lymphocyte culture (MLC) is an *in vitro* test of

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lymphocyte responses (responders) to foreign or non-self lymphocytes incapable of growth (stimulators). The resultant response of the proliferating population is termed mixed lymphocyte response (MLR) (American Society of Histocompatibility and Immunogenetics Laboratory Manual (ASHI), 1989, p. 339).

Lymphocytotoxicity is the presence of complement-dependent antibodies in maternal serum capable of lysing paternal lymphocytes (ASHI, 1989, p. 195).

Leukocyte phenotypes are the different types of leukocytes circulating in maternal peripheral blood (Roitt , et al., 1989. pp. 2.4-2.7).

Salpingitis are infections of the fallopian tube (Cunningham et al., 1989, p. 739).

Decidua is the endometrium of pregnancy. It is shed after pregnancy is completed (Cunningham et al., 1989, p 52-54).

Placentation is the process of forming a cellular structure, the placenta, which is attached to the inner surface of the uterus and is a venue for fetal nourishment (Cunningham et al., 1989, p. 49).

HCG Human chorionic gonadotropin is a placenta hormone produced during pregnancy. It is a diagnostic tool for ectopic gestation. Its increase indicates viable trophoblast and the rate of increase is dramatically altered in ectopic verses normal pregnancy (Cunningham et al., 1989, p. 70).

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Fimbria is the finger-like structure of the fallopian tube proximal to the ovaries (Damjanov, 1993, p. 46).

T lymphocyte is a cell of the immune system produced by the thymus. It is capable of recognizing antigens in association with HLA molecules (Roitt et al., 1989, p. 2.1-2.4).

B lymphocyte is a cell of the immune system that is responsible for producing antigen-specific antibodies (Roitt et al., 1989, pp. 2.1-2.7)

IgG is a major (70-75%) subclass of immunoglobulins. It is a monomeric protein composed of two heavy and two light chains with variable and constant regions (Roitt et al., 1989, pp. 5.2-5.3).

Gravidity is the number of pregnancies irrespective of outcome (Cunningham et al., 1989, pp. 257-258).

Parity is the number of pregnancies that resulted in delivery of a fetus who reached a viable stage of gestation (Cunningham et al., 1989, pp. 257-258).

Non-pregnant controls were females who had tubal ligation and returned for tubal anastomosis.

Pregnant controls were determined to be pregnant through serum pregnancy test (β-HCG) and abdominal ultrasound confirmation.

PHA Phytohemagglutinin is a plant-derived protein that causes non-specific T-lymphocyte proliferation (Roitt et al. 1989, p. 2.9).

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CHAPTER TWO

Review of the Literature

The study centers around three major concepts in the field of reproductive immunology. The first concept addresses the expression of HLA at the feto-maternal interface. HLA are the main determinants of allograft rejection, and their presence or absence on fetal tissue is essential to understanding the nature of maternal acceptance of the fetus. In addition, this study provided MLR data which is determined primarily by the differences in the class II HLA. The second is maternal production of complement-dependent anti-paternal lymphocytotoxic antibody activity. Trophoblastic tissue is considered immunogenic due to paternally derived fetal antigens. Thus, the study of maternal humoral response is important for understanding maternal tolerance to fetal tissue. The third is blood leukocyte populations. Differences in effector cell populations at the implantation site and in the peripheral circulation may play a major role in regulating maternal immune responses to fetal antigens. This study concentrated on detecting any differences in the ratio of maternal leukocyte populations in the peripheral blood that may occur in ectopic gestation, normal gestation and non-pregnant female controls.

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Since the fetus may be considered a successful semi-allogeneic graft in the host mother, a discussion of maternal tolerance to foreign fetal antigens is important in uncovering the mechanisms for fetal survival. As in transplantation immunology, the expression of HLA on both fetal and maternal tissue is central to understanding the nature of the maternal immune response. Moreover, the mixed lymphocyte culture test, the major assay in this study, is a measure of T-lymphocyte activation by mononuclear leukocyte populations as a result of class II HLA disparity. Thus, a detailed review of the involvement of HLA in fetal recognition is essential to a comprehensive analysis of the data.

Maternal tolerance to the implanted fetus may be analyzed using two different approaches. On approach is studying the immunogenicity of fetal tissue and its capabilities of inducing a maternal immune response. Another approach is the maternal immunomodifications that intervene to protect the fetus from the expected adverse rejection reaction.

HLA Expression on the Feto-maternal Interface

An important aspect in determining immunogenicity of fetal tissue is the expression of HLA on the surface of cells that form the interface between mother and fetus. HLA have long been established as the main contributors to graft rejection. Fetal tissue in the early stages of gestation

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lack traditional HLA and, thus, it is immunologically neutral and incapable of eliciting an immune response (Faulk & Temple, 1976; Sunderland et al., 1981). Other studies demonstrate HLA class II antigens on the surface of the amnion which is fetal in its cellular origins and on maternal decidual cells from placenta of women delivering at 36 weeks or later (Sutton, Mason, & Redman, 1983). A study of HLA production in first, second, and third trimester pregnancies show that HLA class I antigens are detected on the extravillous trophoblast. However, maternal immune response to these antigens is not detected (Redman, McMichael, Stirrart, Sunderland, & Ting, 1984). Transformed and normal trophoblastic cell lines induced with interferon-gamma (INF-y) to enhance HLA class I expression are incapable of expressing these antigens (Hunt, Andrews, & Gray, 1987). The expression of HLA class I and II antigens along with β_2 microglobulin on polyploid 2-8 cell-stage pre-implantation embryos is lacking, thus rendering more evidence regarding the inability of early fetal tissue to express traditional HLA (Desoye, Dohr, Motter, Winter, Urdl, Pusch, Uchanska-Ziegler, & Ziegler, 1988).

The appearance of HLA class II antigens is dependent on the gestational age of the conceptus. Early fetal tissue lack HLA class II antigens, but by the eleventh week the placenta and amniochorion demonstrate reactivity to anti-HLA class II antibodies. The cells of the

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placenta with the greatest reactivity to HLA class II antibodies are macrophages (Sutton, Gadd, Mason & Redman, 1986).

Most of the previous investigations concentrated on the extra-embryonic tissue that forms the point of contact with the mother. The trophoblastic tissue constitute the fetal contribution to the feto-maternal interface and is bathed with maternal blood. Early trophoblastic tissue lack the expression of HLA I and II both of which are needed for the classic rejection reaction. While some fetal tissue have certain non-traditional HLA class I antigens, most early trophoblastic tissue lack the class II antigens that produce the more rigorous immune rejection response.

The absence of HLA antigens, however, does not solely explain fetal survival. The trophoblast itself expresses antigens other than HLA antigens that are transplantation-like antigens and are capable of eliciting an immune response (Faulk & McIntyre, 1981).

Maternal tissue in close proximity to the implantation site such as epithelial glands also lack HLA class II antigens but some trophoblastic fetal antigens are expressed on these tissue. Thus, the mother may initiate a mechanism whereby she loses recognition of self either through the lack of HLA antigens or the presence of active suppression. A large number of endometrial granulocytes are present in the epithelial glands near the

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implantation site and may play a role in the down-regulation of maternal HLA on glandular epithelial cells (Johnson & Bulmer, 1984).

Further characterization of maternal HLA class II antigens in the fallopian tubes of normal and ectopic pregnant females reveal that in both conditions epithelial cells posses HLA-DR, DP, and DQ, while similar cells from non-pregnant females are lacking HLA-DP and DQ. The fallopian tube and endometrial epithelium react differently in pregnancy. Normal pregnancy endometrial epithelial glands are negative for HLA class II antigens (Johnson & Bulmer, 1984) while fallopian tube epithelium expresses these antigens (Bulmer & Earl, 1987).

In summary, class I HLA are expressed on some trophoblastic cells such as the extravillous trophoblast but not on cytotrophoblast and syncytiotrophoblast. The class I HLA on the extravillous trophoblast is unlike the traditional class I HLA in that it is not polymorphic. In addition to the unusual expression of class I HLA, fetal tissue also expresses transplantation-like trophoblastic antigens. Both sets of antigens contribute to the immunogenicity of the fetus. Thus, modifications of the maternal immune system are important to understand the tolerance mechanisms of the mother toward the trophoblastic antigens of the implanted blastocyst.

Lymphocyte Responses and Plasma Factors in Pregnancy

The mixed lymphocyte culture has been widely used in transplantation immunology to identify compatible organ donors. It is a measure of T-lymphocyte proliferation when stimulated with a population of foreign leukocytes. T-lymphocyte activation is the result of disparities in the HLA class II, mainly within the HLA-D region (ASHI Laboratory Manual, 1989). The previous section presented the intimate involvement of HLA in the recognition of the fetus. As a result, the mixed lymphocyte culture assay was applied to identify the effect of HLA disparities on pregnancy outcome. Past studies used two approaches to determine maternal response to fetal antigens: one was the direct use of fetal cord cells as the stimulating population and the other was the indirect method of using paternal cells to study maternal immunity to paternally-derived fetal antigens.

Plasma from pregnant, primigravid women at 12 weeks gestational age has an inhibitory effect on mother-to-child and mother-to-unrelated male MLC's. Fetal plasma, on the other hand, inhibits mother-to-child but does not inhibit mother-to-male MLC's (Kasakura, 1971). Plasma from pregnant females produce similar inhibitory effects on other combinations of mixed lymphocyte cultures between mothers, fathers, newborns and unrelated donors. The lymphocyte responses of the mother and father to fetal lymphocytes are similar. In addition, fetal cells are hyporeactive to

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stimulation with maternal lymphocytes presumably due to the production of *in vitro* factors capable of blocking MLR (Ceppellini, Coppo, Miggiano, Pospisil, Curtoni, & Pellegrino, 1971). Plasma inhibitory effects are not restricted to allogeneic stimulation and were also seen in PHA cultures. PHA responses from non-pregnant women are greater than those from pregnant female. Plasma from pregnant females inhibit the PHA response of lymphocytes from non-pregnant females (Jha et al., 1974).

The inhibitory effects of plasma are due to specific antibodies that block MLR. The importance of these antibodies was first determined in chronic recurrent spontaneous aborters. Women suffering from this condition lack the blocking antibodies that are found in plasma from normal pregnant females. Further characterization of these antibodies demonstrate that they are of the IgG class and not directed against HLA class I antigens. The term "blocking" was given to these antibodies because they are capable of blocking lymphocyte proliferation in a MLC. Thus, one feature of maternal tolerance mechanism is a humoral response that contributes to fetal acceptance. Maternal B-lymphocytes, upon contact with trophoblastic antigens produce antibodies that block allogeneic recognition (Roklin, Kitzmiller, Carpenter, Garovoy, & David, 1976).

Jenkins et al. (1972) showed that lymphocyte proliferation is affected by the gravidity and parity of the women. Lymphocyte responses from

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multiparous women stimulated with both fetal and paternal lymphocytes are lower than the responses of nulliparous women stimulated with the same cells. The addition of maternal serum has no effect on lymphocyte responses of nulliparous women. The responses of multiparous women were greater without maternal serum. Thus, lymphocyte nonresponsiveness in women is initiated after the first pregnancy. Similar studies show that lymphocytes from pregnant women are less responsive than those from non-pregnant women in MLC's where the stimulator lymphocytes are from unrelated donors. Therefore, lymphocyte nonresponsiveness in pregnant females is not specific to the stimulatory cell type, spouse or unrelated male control (Jones & Curzen, 1973).

Normal pregnant female lymphocyte responsiveness to paternal lymphocyte stimulation is similar to the responses to other male lymphocytes, regardless of the gestational age. Maternal lymphocyte responses are similar throughout pregnancy and the puerperium. However, when pregnancy plasma from the second trimester is added, the MLR is inhibited. The inhibition reaches a maximum at delivery and returns back to normal within the first week postpartum (Herva et al., 1977). Suppressive capabilities of maternal serum start at 29 weeks of gestation and reaches a maximum at 36 weeks (Bissenden, Ling & Mackintosh, 1980). The inhibitory effects are characteristic of plasma and not serum of pregnant

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females suggesting a role for the clotting system in maternal inhibitory activity (McIntyre & Faulk, 1979b). The suppressive capabilities of maternal plasma are partially due to specific antibodies that are found in the eluates from human placenta. The antibodies are not anti-HLA and they inhibit PHA, one- and two-way MLC's. The antibodies are directed against some unknown trophoblastic antigens (Faulk, Jeannet, Creighton & Carbonara, 1974).

Later studies characterized two trophoblastic antigens that elicit maternal humoral responses, trophoblast antigen-1 (TA-1) and trophoblast antigen-2 (TA-2). The former is a protein with some carbohydrate moieties while the latter is predominantly a carbohydrate with little protein (Faulk, Temple, Lovins, & Smith, 1978; McIntyre & Faulk, 1979c). TA-1 and TA-2 induce the production of IgG responses. Rabbit anti-sera prepared from TA-1 and TA-2 inhibit MLR, adding support to the hypothesis that anti-trophoblastic antibodies are responsible for the inhibition of MLC (McIntyre & Faulk, 1979a). Currently, eleven antigens have been characterized on trophoblastic cells. Monoclonal antibodies are present for most of these antigens but there is no antibody that is specific to trophoblast cells with no cross reactivity to other tissue (Anderson, Johnson, Alexander, Jones, & Griffin, 1987). Other types of anti-trophoblastic antibodies are toxic to paternal lymphocytes. These antibodies are reactive with both trophoblast

antigens and antigens on the surface of paternal lymphocytes. The antigens that share this specificity are called trophoblast lymphocyte cross reactive (TLX) antigens (McIntyre, McConnachie, Taylor & Faulk, 1984).

Maternal immunotolerance to fetal antigens also includes idiotypic-antiidiotypic regulation of immune responses. Pregnancy induces the production of site-specific antiidiotypes to HLA-DR antibodies. The sera of multiparous females contain antibodies that are capable of inhibiting lymphocytotoxicity by anti-HLA-DR typing sera. Thus, the plasma of pregnant females contain antibodies that are capable of inhibiting the action of another set of antibodies directed against HLA-DR antigens. Further characterization of the specificities of these anti-HLA-DR antibodies revealed that they are specific to paternal HLA-DR types. The antiidiotypic antibodies show cross reactivity by inhibiting the cytotoxic activity of antibodies from different donors. Autoantiidiotypic antibodies from pregnant females are capable of blocking and binding idiotypic antibodies from other pregnant women (Horini, & Terasaki, 1982; Reed, Bonagura, Kung, King, & Suciu-Foca, 1983). Pregnancy-produced antiidiotypic antibodies react with autologous lymphocytes primed with paternal lymphocytes or unrelated lymphocytes that expressed the same paternal DR specificities. In addition, they are capable of significantly inhibiting MLR. The mechanism by which these antibodies function to protect the fetus is still unclear but it is

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speculated that they block maternal cellular and humoral responses that could adversely effect the acceptance of the fetus (Signal, Butler, Liao, & Joseph, 1984; Suciu-Foca, Reed, Rohowsky, Kung, & King, 1983; Bonagura, Ma, McDowell, Lewison, King, & Suciu-Foca, 1987).

Earlier studies demonstrate the presence of antibodies in the serum of pregnant women that are directed against fetal antigens of paternal origin. The antibodies are missing in the serum of women who suffer from unexplained recurrent spontaneous abortion. These antibodies display *in vitro* cytotoxicity to paternal lymphocytes and are presumed to be necessary for the maintenance of normal pregnancy (Van Rood, Van Leeuwen, & Ernisse, 1958; Payne, 1962; Doughty & Gelsthorpe, 1971). Clinical trials using immunotherapy with paternal lymphocytes show a significant positive correlation between the presence of these cytotoxic antibodies and successful pregnancy. The infusion of paternal lymphocytes in patients with recurrent miscarriages and their subsequent sero-conversion improve their probability of a successful pregnancy as compared to patients who did not sero-convert (Taylor & Faulk, 1981; Mowbray, Gibbings, Liddell, Reginald, Underwood, & Beard, 1985). However, the presence of these antibodies is not essential for the success of pregnancy in general. The detection of cytotoxic antibodies is variable and depends on the parity, gravidity, and the time of pregnancy at which the blood was collected (Regan & Braude, 1987;

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Regan, Braude & Hill, 1991). Other studies also report that cytotoxic antibodies are not necessary for the maintenance of a successful pregnancy (Hasegawa, Takakuwa, Adachi & Kanazawa, 1990).

In conclusion, during normal pregnancy B-lymphocytes are induced by trophoblastic antigens to produce antibodies. T-lymphocytes are prevented from proliferation due to the action of anti-trophoblastic antibodies that prevent T-cell activation and the subsequent cytotoxic reaction to the implanted fetal tissue.

Immunoendocrinologic Regulatory Factors

Non-specific factors such as pregnancy hormones also contribute to immunoregulation. The endocrine effects on immune function may be a key factor in understanding fetal survival.

Progesterone which is produced in high concentration during pregnancy is considered and immunosuppressant and inhibits both allogeneic and mitogen lymphocyte cultures *in vitro* at concentrations much higher than those encountered in pregnancy. Progesterone also functions to up-regulate estrogen receptors (Sitteri, Febres, Clemens, Chong, Gondes, & Stites,1977; Clemens, Sitteri, & Stites, 1979). T-lymphocytes posses estrogen receptors and when the receptors combined with estrogen they inhibit suppressor T-cell activity (Paavonen, Anderson, & Adlercreuts, 1981;

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Feigen, Fraser, Peterson, & Dandlikes, 1978). Thus, the interaction of both these hormones influence humoral and cellular immune responses during pregnancy. Furthermore, a hormone-like molecule is produced *in vitro* as a result of allogeneic stimulation of lymphocytes. The hormone which resembles chorionic gonadotropin is referred to as immunoreactive chorionic gonadotropin (irCG). The source of this molecule is unknown but it may play a role in regulating implantation and protecting the fetus from rejection. This protection is possibly accomplished through the inhibitory effects high concentrations of irCG has on allogeneic activation of T-cells (Harbour-McMenamin, Smith, & Blalock, 1986). Conflicting results from other studies show that some pregnancy hormones (estrone, estradiol, estriol, progesterone, HCG, and hydrocortisone) are incapable of inhibiting DNA synthesis in a mixed lymphocyte reaction unless added at supra-physiological concentrations. Physiological concentrations found in normal pregnancy fail to inhibit allogeneic and mitogen-induced lymphocyte responses. Lymphocyte responses inhibited by high concentration of estradiol and HCG are capable of resuming DNA synthesis once they are washed and the effects of the hormones are removed (Schiff, Dominique, Merger, & Buckley, 1975).

The effects of the endocrine system in the regulation of immune responses in pregnancy are ample. Hormones from pregnant females

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present dichotomous regulatory effects on immune function depending on the concentration and the time at which they are secreted.

Leukocytes at the Implantation Site

The immune cells which reside at the implantation site of intrauterine and ectopic pregnancy are critical in determining the maternal immune response. Characterization of leukocyte populations at the implantation site may support the hypothesis that ectopic pregnancy has unique immunologic features. The data relating to this concept is limited by the invasiveness of the procedures necessary to obtain accurate and representative samples. Most efforts have concentrated on leukocyte populations in the placenta of normal pregnancy. Few studies have reported on differences between normal and pathologic pregnancies.

Immunohistochemical techniques reveal that term decidua has a large number of HLA-DR positive macrophages in the basalis layer. The majority of T-cells within the basalis layer express the T8 antigen indicating that the cells are of the suppressor/cytotoxic subset. In first-trimester decidua, the layers closer to the trophoblast contain a large number of "irregular macrophages" similar to the term decidua. However, in the deeper uterine layers, macrophages are less prominent. Immature T-cell populations are more commonly encountered in the deeper tissue. These immature T-cells

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are missing in the superficial tissue close to the fetus. While the function of these cells still remains unknown, the investigators speculate that they provide immunosuppressive activity capable of inhibiting traditional immune responses (Bulmer, Johnson, & Bulmer, 1987).

Significant NK activity is found in the decidua, and it is restricted to CD56+/CD3-/CD16- large granular lymphocytes (LGLs). In contrast, peripheral blood mononuclear cells have a minor population that fit this antigenic structure and they exhibits weak NK activity. Also, there are possible interactions between leukocyte populations and cytokine activity which is presumed to impact the immunoregulation of pregnancy (Ferry, Starky, Sargent, Watt, Jackson, & Redman, 1990). There is a high ratio of CD3/HLA-DR+ lymphocytes in the decidua of recurrent spontaneous aborters as compared to ectopic and normal pregnancy. There is no difference in other leukocytes subsets that were evaluated by the study. The distribution of leukocyte subsets are independent of the embryo implantation status and may be primarily affected by hormonal and cytokine activity (Maruyama, Makino, Sugi, Matsubayashi, Ozawa, & Nozawa, 1992). Further reports on the same phenomena show higher NK activity in the decidua of a normal term placenta as compared to maternal peripheral blood. Moreover, progesterone receptors are necessary for the sustaining the activity of NK cells (Roussev, Higgins, & McIntyre, 1993).

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The placenta possesses a unique intra-uterine immune structure. The functionality of placental leukocyte populations are different from those found in matching maternal and fetal peripheral blood (Goldsobel, Ank, Spina, Giorgi, & Stiehm, 1986). There is a difference between the distribution of natural killer (NK) cells in first-trimester human decidua and the matching maternal peripheral blood NK population. (Manaseki & Searle, 1989).

Lymphocyte populations from mid gestation have a large number of CD3+ cells in the peripheral circulation. CD16+ and CD20+ are comparable in the blood and placenta of pregnant women. CD56+ are present in greater quantities in the placenta. There were few CD3+ cells in the placenta, and their role as it relates to fetal immunobiology is not clear (Erbach, Semple, Milford, Goguen, Osathanondh, & Kurnik, 1993).

The T-lineage cells bearing immature T-cell markers and E-rosette receptors found in the first trimester of intrauterine pregnancy are not present in ectopic gestation. Ectopic pregnancy lacks the normal specialized T-cell populations seen in normal pregnancy. Instead, ectopic pregnancy is characterized by the presence of a smaller classic T-lymphocyte population at its implantation site. Ectopic gestation mimics intrauterine pregnancy in the presence of a large number of macrophages at the implantation site. While the macrophages at the ectopic implantation site express HLA-DR,

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macrophages in the fallopian tubes of normal pregnant and non-pregnant females do not express HLA-DR specificities (Earl, Lunny & Bulmer, 1987). Moreover, endometrial epithelial tissue goes through some phenotypic changes in early intrauterine gestation such as the loss of HLA class I determinants (Johnson & Bulmer, 1984). Tubal epithelial tissue, on the other hand, does not show these changes in ectopic pregnancy (Bulmer & Earl, 1987).

Studies on maternal cellular and humoral responses are founded on data from studies with variable designs. Therefore, analysis of the literature must be done with careful attention to the details of the experimental designs. Some researchers use serum, others use plasma. There was always a difference in the gestational age of the pregnant women. The number of patients used was variable even within one study and the mathematical representation of MLC data was different among various studies. In general the experimental design of most studies differed sufficiently and rendered a body of data that is inadequate to construct a concrete theoretical model. The phenomena regarding the inhibitory effects of pregnancy plasma on mixed lymphocyte culture depends on the mathematical equation used to present MLC data (Park, Edwin, Scott, & Ware-Branch, 1990). Coulam (1992) reviewed data from a number of immunological assays including MLC and their application in reproductive

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immunology and concluded that results were variable from these assays and depended on the duration of pregnancy, number of pregnancies, and multiple factors within the experimental designs.

Maternal immunoregulatory mechanisms during pregnancy are diverse including cellular, humoral, and endocrinological factors. The interdependence of many of these mechanisms, collectively, result in the protection of the fetus. While many of the facts surrounding maternal immunotolerance towards fetal tissue have been described, the exact cascade of events following intrauterine implantation that leads to tolerance of the fetus is not known. Therefore, studies that characterize maternal immune function in pathologic pregnancies where the intact network of maternal regulatory factors is disrupted may reveal a wealth of information regarding the immunoregulation of normal intrauterine pregnancy.

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CHAPTER THREE

Methods

Rational

The study followed the design of other immunologic studies aimed at understanding clinical conditions such as unexplained spontaneous abortions. None of the literature provides conclusive data regarding the relationship between maternal immunity and ectopic gestation. The design of this study was exploratory in nature and aimed at providing greater characterization of maternal immunologic responses in ectopic pregnancy. Moreover, since ectopic gestation may be considered a unique natural experiment where the implantation site is altered, the research may aid in understanding the role of the uterus in the maintenance of successful pregnancy.

It is unknown whether ectopic pregnancy exhibits the same immunologic features as normal pregnancy. The evaluation of ectopic gestation will address the humoral and cellular immune responses and how they compare to the immunologic features of normal pregnancy. The research objectives are focused on the alterations in maternal immune response that result from a change in the implantation site of the fetus.

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Statement of Research Objectives

1- Is there a difference in the mixed lymphocyte response of females with any of the three pregnancy conditions to their male spouse versus unrelated male controls? This question addresses the role HLA sharing between mating couples play in pregnancy outcome. It is aimed at determining whether females with ectopic pregnancy have a disproportionate sharing of HLA alleles with their male spouses as compared to unrelated males.

2- Is there an inhibitory or stimulatory effect of plasma from the three pregnancy conditions on the mixed lymphocyte response? One of the immunoregulatory features of pregnancy is the presence of MLR blocking factors in maternal plasma. A study of plasma effects in ectopic pregnancy will determine whether this regulatory feature is characteristic of normal pregnancy only, or is it modified with a change in the implantation site.

3- Is there a difference in the plasma effects on PHA-stimulated lymphocyte cultures and male-to-male MLR? This question addressed the specificity of plasma effects. Are these effects only seen in female lymphocyte responses or does it extend to male lymphocyte responses? And, are the plasma effects specific to MLC or, are they seen when the mode of lymphocyte stimulation changes?

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4- Do females with ectopic gestation have any complement-dependent anti-paternal lymphocytotoxic antibodies? *In vitro* cytotoxic antibodies are another probable immunoregulatory feature of normal pregnancy. It is unknown whether they are detected in ectopic pregnancy.

5- Is the maternal peripheral lymphocyte phenotype different among the three pregnancy outcomes? This question expands on the finding that leukocyte populations are different at the normal versus tubal implantation site. It investigates whether there are differences in circulating leukocyte populations.

<u>Hypothesis</u>

The general hypothesis was formulated as a substantive hypothesis that was then sub-divided into statistical hypotheses (Kerlinger, 1986).

<u>Substantive Hypothesis</u>

There is a difference in the immune function of ectopic pregnant, normal pregnant and non-pregnant females.

<u>Statistical Hypotheses</u>

Mixed lymphocyte culture assay:

1) H_a: There is a difference in the mean %RR of female lymphocytes among the three conditions with no plasm a added.

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H$_o$: There is no difference in the mean %RR of female lymphocytes among the three conditions with no plasma added.

2) H$_a$: There is a difference in the mean %RR of male spouse lymphocytes among the three conditions with no plasma added.

H$_o$: There is no difference in the mean %RR of male spouse lymphocytes among the three conditions with no plasma added.

3) H$_a$: There is a difference in the mean SI of female MLR's among the three conditions, two stimulating cell types, and five plasma types.

H$_o$: There is no difference in the mean SI of female MLR's among the three pregnancy conditions, two stimulating cell types, and five plasma types.

4) H$_a$: There is a difference in the mean SI of male-to-male MLR's among the five plasma types.

H$_o$: There is no difference in the mean SI of male-to-male MLR's among the five plasma types.

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Mitogen stimulation assay:

H_a: There is a difference in the mean PHA SI of female lymphocytes among the three pregnancy conditions and five plasma types.

H_o: There is no difference in the mean PHA SI of female lymphocytes among the three pregnancy conditions and five plasma types.

Cytotoxicity assay:

H_a: There is a difference in maternal cytotoxic activity against paternal lymphocytes among the three pregnancy conditions.

H_o: There is no difference in maternal cytotoxic activity against paternal lymphocytic antigens among the three pregnancy conditions.

Lymphocyte phenotypes :

H_a: There is a difference in the phenotype ratios among the three pregnancy conditions.

H_o: There is no difference in the phenotype ratios among the three pregnancy conditions.

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Sampling

A sample of convenience consisting of patients and controls was selected from Portsmouth Naval Hospital (PNH). All patients signed an informed consent which remains in their file at the Naval Hospital. The experimental protocol was reviewed and approved by the Committee for Protection of Human Subjects at the Naval Hospital in Portsmouth, Virginia.

All the participants were classified in accordance with their pregnancy condition. Upon final analysis there were twelve ectopic, nine normal pregnant, and ten multiparous non-pregnant females. The average ages were 32 years for the non-pregnant group, 29 years for the pregnant group with a mean gravidity of 2 and parity of 0.7, and 28 years for the ectopic group with a mean gravidity of 2.8 and parity of 1. The gestational age ranged for normal pregnant females from 4 - 7 weeks with a mean of 6 weeks, and for ectopic pregnant females from 3 - 8 weeks with a mean of 5 weeks. The general health of the participants was good with no known viral or bacterial infections and no known conditions that may cause immunodeficiency. Non-pregnant controls consisted of females who had received tubal ligations and returned to the hospital for tubal anastomosis. Three unrelated male controls were included in the MLC and cytotoxic assay.

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Method Reliability and Validity

Reliability

Precision of all methods was tested by replicating the measurement of each data point. In the mixed lymphocyte culture, the same cell reaction was tested in three separate wells. The mean coefficient of variation was 0.588%. Furthermore, the intensity of the MLR is dependent on the number of non-identical HLA haplotypes. Lymphocyte populations with two identical HLA haplotypes have a lower MLR than populations with one identical HLA haplotype. MLC's with non-identical haplotypes have the greatest MLR's. Hence, the mixed lymphocyte culture is reliable in detecting differences in HLA and is a functional measure of cellular immunity (Bain, Vas, & Lowenstein, 1964; Bach & Hirschhorn, 1964).

Each reaction in the cytotoxic assay was set up in duplicate and there was no significant difference between the first and the second duplicated wells indicating that the assay was precise. The leukocyte phenotype assay was run in duplicate using two different methods and there were no differences in the results obtained by the two determinations. Moreover, all the assays used in this study followed the protocols of the American Society of Histocompatibility and Immunogenetics (ASHI Laboratory Manual, 1989) which are approved by the Food and Drug Administration for patient testing and diagnosis.

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Cellular, mitogen, and mitomycin-C quantities were subjected to dose-response analyses to determine the most effective combinations to yield optimal results (Appendix A). The number of cell washes, the effect of collection and the storage of cells were also assessed in the appropriate assays to control for variability.

Validity

Validity of all experiments was demonstrated by using internal controls. These control cultures were introduced into each assay to determine if the cells were capable of responding in the expected fashion under control conditions. In the mixed lymphocyte culture, an autologous one-way MLC (both stimulator and responder cells are from the same participant) was introduced to give a base-line reading for each of the responder cell types. Individual lymphocytes do not proliferate when challenged with an autologous population of lymphocytes. All reactions that showed proliferation in the autologous, base-line, control and read higher than mixed lymphocyte reactions were dropped from the final analysis. The mitogen PHA was used as a positive control to demonstrate the ability of cultured lymphocytes to respond to non-specific, polyclonal, stimulation. Any assay where the control wells showed general inhibition with mitogen stimulation were dropped from the data set. PHA added to mitomycin-treated cells served as a negative control and demonstrated that these cells

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were incapable of proliferation. The media quality control described in the methods section ensured that data was not misrepresented due to contamination with fungal or bacterial elements.

The validity of the cytotoxic data was also verified through the use of positive and negative controls. A commercially prepared antibody to human lymphocytes (Pelfreeze) was used as a positive control and two wells including cells, media, and complement were used as a negative control.

Studies done at Walter Reed Medical Center were determined to be valid in accordance with the policies of the laboratory which follow the mandated governmental regulation for quality control.

Assumptions and Limitations

Assumptions referred to in this section are those conditions that were beyond the control of the investigators, and were assumed to be constant through the study. Limitations are the various restrictions and controls that were selected *a priori*.

Assumptions:

1) All pregnancies were assumed to be the product of mating between the couples identified as husband and wife.

2) Control and experimental groups were free of any medical or physical conditions that may effect their immune responses.

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3)   All cultures were free of contaminants and all experimental systems were sterile.

4)   Paternal antigens are capable of eliciting the same maternal immune response as fetal antigens of paternal origin.

Limitations:

1)   The study population was non-random which limited the use of inferential statistics.

2)   All the participants were drawn from one population of active duty, and reserve naval personnel and their dependents.

3)   Pooled plasma and cells were the product of no more than three controls.

4)   The definition of immune function was restricted to the measure of the mixed lymphocyte responses, the lymphocytotoxic activity in maternal serum, and the leukocyte phenotypes.

5)   No unrelated females were introduced into the experimental design to control for the response of male spouse lymphocytes to their respective female lymphocytes.

6)   The leukocyte phenotype enumeration was limited to a selected number of common types.

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Assay Techniques

One-way Mixed Lymphocyte and Mitogen Stimulation Cultures

Objective. To determine differences in lymphocyte proliferation (using both allogeneic and mitogen stimulation) between the various pregnancy outcomes due to HLA antigen sharing; and to determine any inhibitory or stimulatory effects of plasma obtained from the different pregnancy conditions on the MLR.

Background and principle. The mixed lymphocyte culture was first encountered when a group of scientists noticed the development of large immature cells when blood samples from two leukemic patients were accidentally mixed (Scherk and Donnelly, 1961). Further studies (Bain, Vas, & Lowenstein, 1964; Bach & Hirschhorn, 1964; Bach, Bach, & Joo, 1969) revealed that a hyper-proliferative response is produced when two populations of lymphocytes with different HLA class II antigens are mixed. The phenomena was termed a mixed lymphocyte response (MLR). Mitogen stimulation is an assay that tests T-cell function. It is a method for the polyclonal activation of T-lymphocytes (ASHI, 1989, p. 91).

Procedure. Venous blood samples from female participants, their male spouses, and unrelated controls were collected by the Naval Hospital staff using vacutainer tubes containing the enrichment media Acid Citrate Dextrose solution A (Vacutainer). Upon receipt, the tubes were directly

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centrifuged at 700xg (Intl. Equip. Co.) for 10 minutes. Plasma from female participants and their spouses was sterilized through a 0.2 μm filter (Schleicher and Schnell). Plasma from unrelated male controls was pooled in equal amounts and filter sterilized.

The buffy coat from each sample was collected into a sterile 50 ml conical tube (Costar) using a sterile Pasteur pipette. Ten milliliters (10 mls) of the buffy coat was loaded onto a Ficoll-Hypaque separation column composed of Histopaque 1119 (5 mls) and Histopaque 1077 (5 mls) (Sigma) and spun for 30 minutes at 700xg. Mononuclear cells were collected using a sterile Pasteur pipette and placed in a clean sterile 50 ml culture tube. The cells were washed once with 50 mls of RPMI 1640 media (Gibco) buffered with HEPES and containing 2 mM L-Glutamine, 100 units of penicillin/ml, 100 μg/ml of streptomycin, 0.25 μg amphotericin/ml, 50 μg of gentamicin/ml (Sigma), and complemented with 20% AB heat-inactivated human serum (Pelfreez). All media stored longer than thirty days without use was replenished with 2 mM L-Glutamine (Gibco).

The cell suspensions were then centrifuged at 700xg for 10 minutes and the supernatant discarded. The cells were re-suspended in 10 mls of RPMI media containing the commercial serum. A cell count was performed using a hemocytometer (Baxter) with Trypan Blue (Sigma) and the cell concentration was adjusted to 1×10^6 viable cells/ml. Stimulator cells were

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produced by incubating 5 mls of the adjusted cell suspension with 0.5 mg/ml mitomycin-C (Sigma) for 30 minutes at room temperature. The cells were then washed twice with 50 mls of media to remove residual Mitomycin-C. Microtiter round-bottom plates (Costar) were filled with 50 μls of responder cells and 50 μls of non-proliferating (mitomycin-treated) stimulator cells in triplicates according to the diagram in Figure 5. The culture system was incubated in 5% CO_2 at 37°C for seven days. On day four 50 μls of PHA (Gibco) were added to the designated row. On day six 20 μls of 50 uCi/ml H^3-thymidine (NEN Research Products) was added to all wells. On day seven the cells were harvested with a cell harvester (Cambridge technologies Inc.) onto fiber glass paper (Cambridge Technologies Inc.). The cells were bathed with distilled water which caused their lyses due to its low osmolality. The released DNA with the incorporated radioactive isotope was trapped on the fiber glass paper. The rinse and aspiration procedure was repeated five times. The fiber glass tabs containing the cellular DNA were then transferred to scintillation counting vials (Wheaton Scientific). Three milliliters of counting solvent (Research Products Intl. Corp.) were added to each vial. The vials were counted using a scintillation counter (Beckman). Data was entered into a Lotus 1-2-3 (Lotus Corp.) spread sheet (Appendix B).
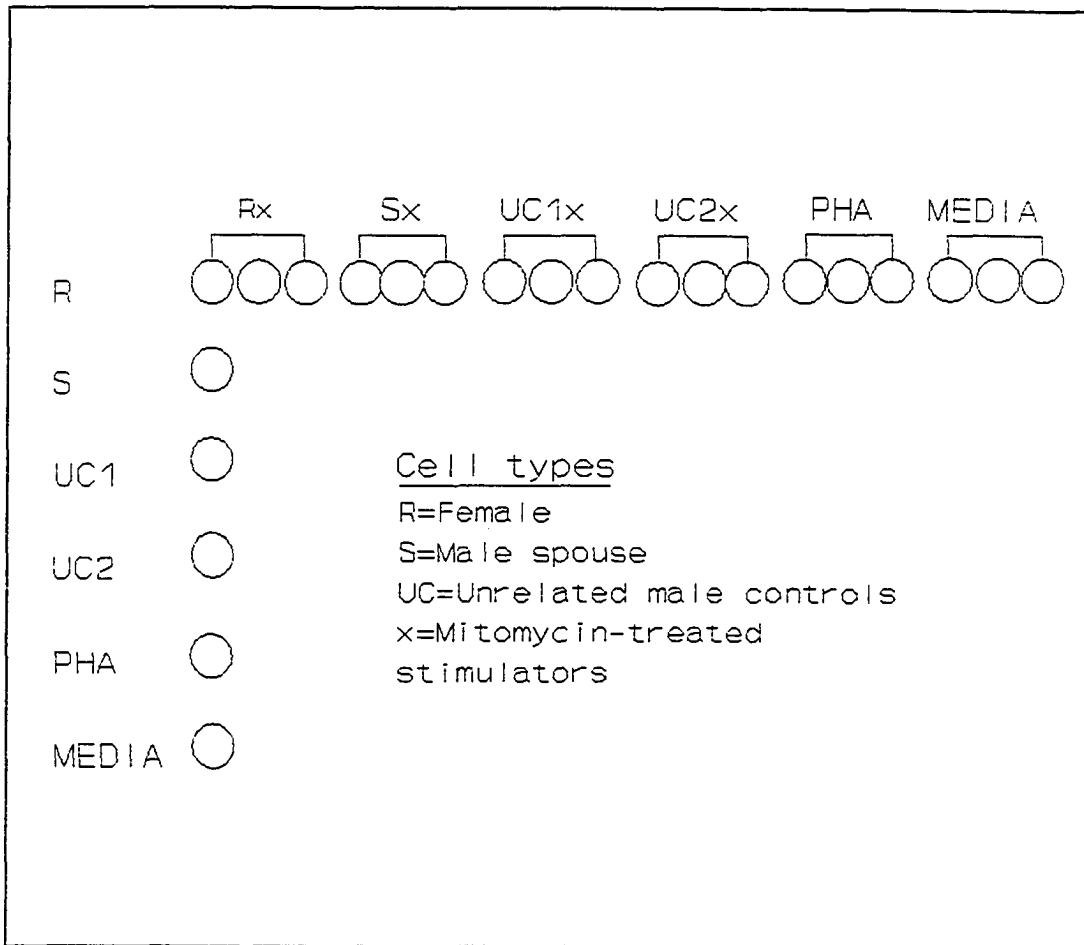
50

Figure 5. Mixed lymphocyte culture plate arrangement.

To test for plasma effects, four identical plates were included for each subject. The first plate contained no plasma. The second plate had pooled plasma from unrelated male donors to serve as a control for the general effects of plasma, including freshness. Autologous plasma from the female subjects were added back to their MLC in the third plate. The fourth plate was a cross-match plate in which plasma from ectopic pregnant females was added to MLC's from normal and non-pregnant females. Alternatively, MLC's from ectopic pregnant females received plasma from normal pregnant females.

Media Quality Control. Each batch of complete media (RPMI + Antibiotics + Heat-inactivated AB serum) was tested for contamination. The media was visually inspected for turbidity, gram stained and cultured in sheep blood and MacConkey agar (BBL). Another set of cultures was performed on cell suspensions immediately before they were transferred to microtiter plates. In addition, a sample from each lymphocyte suspension was cultured in PPOL broth over-night, transferred to PPOL agar (Remel), and then incubated along with the corresponding MLC for seven days.

Any contamination present during or at the end of the seven days resulted in the deletion of the MLC from the study. A total of 12 MLC's were rejected based on these quality control criteria.

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Anti-paternal Lymphocytotoxicity

Objective. The objective of this experiment was to determine the presence or absence of maternal anti-paternal lymphocytotoxic antibodies in the plasma of females with ectopic pregnancy.

Background and principle. The lymphocytotoxicity test is routinely used for HLA-A,B,C and DR antigen typing. The first method was miniaturized by Terasaki (1964). The National Institutes of Health standardized the procedure, while Amos (1965) modified the procedure and added a wash step to the cell-serum wells after incubation. The wash step is necessary to remove non-specific complement-activating components. The principle of the test is to detect maternal complement-dependent cytotoxic antibodies against paternal lymphocytes through lymphocyte lysis.

Procedure. One microliter of the adjusted lymphocyte suspension (1×10^6 cell/ml) from the male spouse, male controls, and females was obtained from initial separation step in the mixed lymphocyte culture assay and transferred to a Terasaki microtiter plate (Robbins Scientific) with wells containing 1 μL light oil (Sigma). These cells were then incubated for 30 minutes at room temperature with plasma from the female spouse. Two negative control wells were included, one with the lymphocytes of the female and her own plasma and another with female lymphocytes and complement. A positive control well was set-up containing female cells, a

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commercially prepared anti-lymphocyte antibody (Pelfreez) and complement. The plate arrangement is shown in Figure 6. All the wells were washed three times with phosphate-buffered saline (PBS). Five microliters of freshly thawed rabbit complement (Pelfreez) was added to each well. After the wash, the system was incubated for 1 hour at room temperature. Filtered 5% aqueous Eosin Y (Sigma) was added to each well and incubated for 3-5 minutes at room temperature. Five microliters of buffered formaldehyde (pH = 6.8-7.5) were added at the end of the incubation period. A cover slip was placed over the entire plate and the cells were allowed to settle for 15 minutes before reading. Each well was read under an inverted phase microscope (American Optics) at 150x. Dead cells lysed by the action of antibodies in maternal serum or the positive control appeared dark, diffused, granular, and non-reflectile.
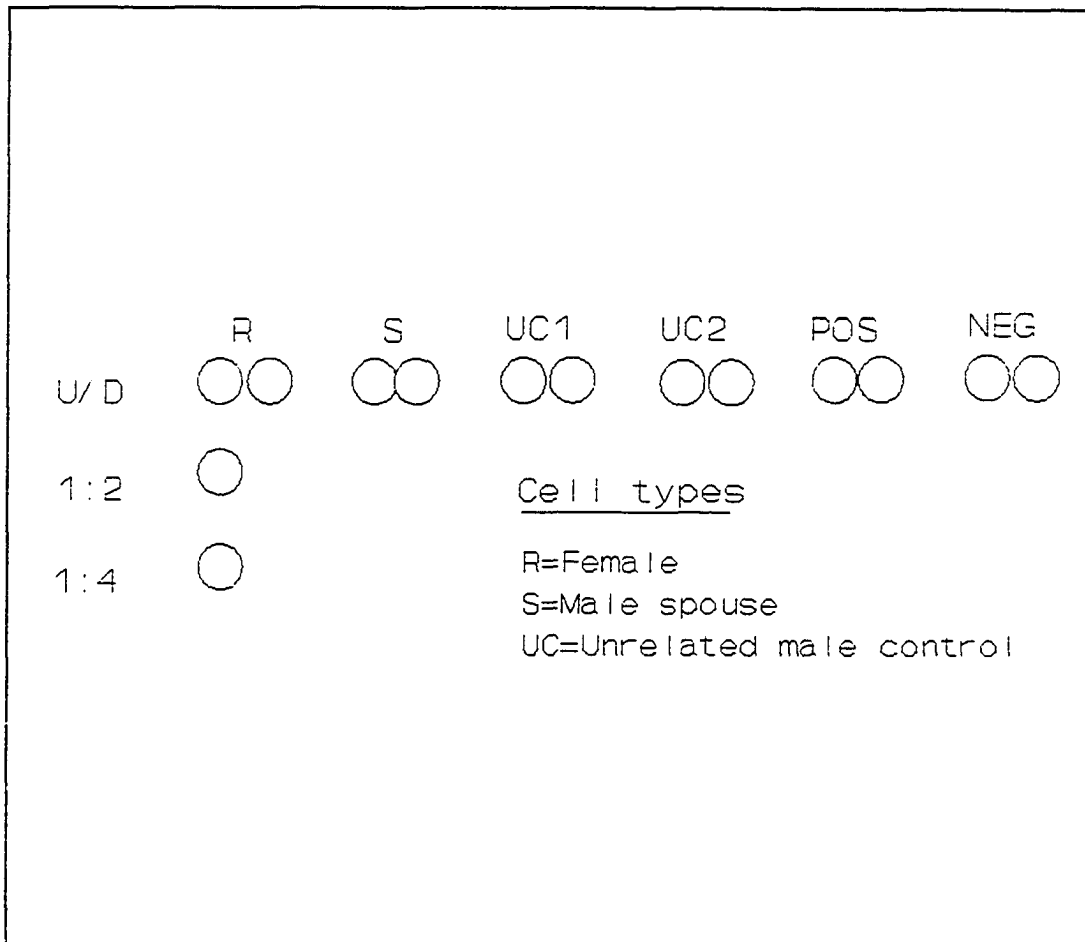
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Figure 6. Anti-paternal lymphocytotoxic assay plate arrangement. The rows represented maternal plasma (un-diluted, 1:2 and 1:4 dilutions).

R   S   UC1   UC2   POS   NEG

U/ D

1 : 2

1 : 4

Cell types

R=Female
S=Male spouse
UC=Unrelated male control

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The unaffected cells looked shiny and their membrane integrity was round and reflectile. The reactions were rated as follows (ASHI, 1989, p. 199):

Score	% Cell Death	Interpretation
1	0 - 10	Negative
2	11 - 20	Borderline negative
4	21 - 50	Weak positive
6	51 - 80	Positive
8	81 - 100	Strong positive
0	Unreadable	None

Peripheral Blood Leukocyte Phenotyping by Flow Cytometry

Objective. The objective of this experiment was to determine whether any of the three conditions had a different number of circulating leukocyte phenotypes.

Background and principle. Flow-cytometry is a cell sorting method in which fluorescent monoclonal antibodies to certain cell markers are used to identify the different leukocyte phenotypes in peripheral blood. The method is capable of measuring and differentiating cells based on size, shape, cytoplasmic granularity, pigment content, DNA/RNA content, and chromatin shape. The sample is a cell suspension that is stained with

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fluorescent conjugated antibodies. Light and photomultipliers are then used to identify structure and staining patterns (ASHI, 1988, p. 238).

Procedure. Blood samples were sent to Walter Reed Medical Center and stored at room temperature until processing. Whole blood was stained by the direct method separately with each fluorochrome labeled monoclonal antibody (fluorescein isothiocyanate or phycoerythrin) (Becton-Dickinson). Fifty micro-liters of blood, 50 μl of phosphate buffered saline (PBS), and 10 μl of each type of conjugated antibody were mixed in a 12mm x 75mm tube (Falcon) and incubated at 4°C for 15 minutes in the dark. Appropriate isotype negative controls (without the conjugated antibodies) were included with each patient sample.

The stained cells were washed with 3.5 ml of PBS containing 0.01% sodium azide and centrifuged for three minutes at 1500 rpm (Beckman). All but 50 μl of the supernatant was discarded. The red blood cells were lysed in the remaining cell pellet. After the last wash following lysis the remaining cells were resuspended with 1% paraformaldehyde in PBS and analyzed by flow-cytometry (Facstar Plus Cell Sorter or the Coulter Epics Elite Cell Sorter). The results were expressed as a ratio of the total leukocyte population that the flow cytometer gated for analysis.

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Data Manipulation and Statistical Analysis

Mixed Lymphocyte Cultures

The MLC and PHA experiments produced interval data. The basic unit of measurement for the MLC was counts per minute (CPM) read by the scintillation counter. The counts were transferred to a Lotus 1-2-3 spread sheet. The spread sheet was constructed to allow mathematical manipulation of the CPM in accordance with the guidelines set by the American Society of Histocompatibility and Immunogenetics Laboratory Manual (1989). The first manipulation termed the Stimulation Index (SI) was a proportional representation of CPM from each mixed lymphocyte culture to the CPM from the autologous culture. It was calculated according to the formula: RSx (or RUCx) / RRx; where R is the viable female responding cell, Rx, Sx, UCx are the mitomycin-treated female, male spouse, and male control cells, respectively. Both RSx and RUCx represent CPM from a mixed culture whereas RRx represent CPM from an autologous culture of female cells responding to an identical population of mitomycin-treated cells from the same female. Autologous cultures were designated as the background or baseline against which each mixed lymphocyte culture was tested to produce the SI. The unrelated control stimulation index was calculated by averaging the three SI's produced by the female to each of the three controls. Mathematically, the control MLC's were calculated as

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follows: (RUCx1/RRx + RUCx2/RRX + RUCx3/RRX) / 3; where RUCx1/RRx is the SI of the female to the first unrelated control and so forth.

A different manipulation of the same CPM from the same MLC data produced the percent response rate (%RR). Response rates represent the rate a female responds to her husband relative to the controls. Hence, a 100% RR means that the response of the female to her husband is equal to her response to the controls. Response rates higher than 100% indicate a greater female response to her husband than to controls (ASHI, 1989). The mathematical representation of this rate is:

$$\{ RSx - RRx / [(RUCx1 + RUCx2 + RUCx3/3)] - RRx \} * 100$$

Mitogen Stimulation Data

The PHA simulation experiment is a standard assay for determining the non-specific, polyclonal activation of T-cells (Jha et al., 1974). PHA is also used as a control to demonstrate that mitomycin-treated cells are incapable of proliferating. The method of simulation differs from the MLC since a plant-derived mitogen is used to elicit a lymphocyte response. The test culture consisted of mitogen plus the responding viable cells, while the control cultures were the viable cells in media alone.

Lymphocytotoxicity Data

Lymphocytotoxicity data is determined by measuring cell death. It is ordinal in nature. The positive and negative controls were assessed first to

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determine the success of the assay and patient readings followed. Lymphocytotoxicity was determined after the addition of maternal plasma to male spouse, male control, and autologous cells.

Leukocyte Phenotypes

Data from this assay produced a ratio for each phenotype to the total lymphocytes gated for counting. An average of each phenotype was calculated and a one-way analysis of variance was performed to detect any differences in the distribution of phenotypes among the pregnancy conditions.

Statistical Analysis

All statistical analyses were performed using SYSTAT software (SYSTAT Inc.) The sampling process and sample size violated the basic requirements for inferential statistics. Since the sample was not randomly selected a Bartlett Chi-square test for the homogeneity of group variances was performed and the hypothesis of no homogeneity of group variances was rejected ($p < 0.05$). The test indicated that the samples were at least drawn from homogeneous populations with respect to their variances (Daniel, 1987). All the parametric statistical procedures were used to describe the sample under investigation and not to provide population inferences.

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H_o: There is no difference in the mean %RR of female or male lymphocytes among the three conditions. Mean %RR obtained from the MLC assay were analyzed using a factorial model analysis of variance (ANOVA) followed by a one-way (ANOVA). Basic statistics were calculated for %RR including means and standard errors about the mean. The female responses to her male spouse cells were analyzed followed by the responses of the male spouse to the lymphocytes of his female spouse.

H_o: There is no difference in the mean SI of female MLR's among the three pregnancy conditions, two stimulating cell types, and five plasma types.

The data was categorized into three independent variables: pregnancy condition, plasma type, and stimulating cell type for the factorial design. Following that, the various treatments were tested using a one-way analysis of variance. The ANOVA was set to exclude any unequal cells. Tukey's honestly significant difference (HSD) for multiple comparison of means was performed to test for the null hypothesis that all the means produced by the treatment were equal. The same statistical analyses were carried out using the %RR as a dependent variable.

H_o: There is no difference in the mean SI of male-to-male MLR's among the five plasma types.

The effects of plasma were also assessed using the previous

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statistical techniques on the SI's of unrelated male controls stimulated with male lymphocytes.

H_o: There is no difference in the mean SI of PHA stimulation of female lymphocytes among the three pregnancy conditions and five plasma types. Data from the responses of female lymphocytes to PHA was analyzed using a one-way ANOVA to test the effect of pregnancy condition and plasma type on PHA stimulation index.

H_o: There is no difference in maternal cytotoxic activity against paternal lymphocytic antigens among the three pregnancy conditions. Data from this assay consisted of ratings of each microscopic field. The maternal lymphocytotoxic activity was grouped by condition. Other than the positive control, none of the treatments showed evidence of any cell death or lymphocytotoxicity. Since all of the experiments yielded the same rating of "1" or "negative" for cell death, no statistical analysis was necessary.

H_o: There is no difference in the phenotypes ratio among the three pregnancy conditions. Phenotyping ratios were averaged for each type and analyzed using a one-way ANOVA to determine if there is any difference in the pools of circulating lymphocytes in each of the three pregnancy conditions.

CHAPTER FOUR

Results

Analysis of statistical output

The factorial model ANOVA using the SI as the dependent variable indicated that "pregnancy condition" is a statistically non-significant estimate of the SI ($F = 2.503$ and $p = 0.08$). Hence, "pregnancy condition" alone cannot explain the variability in lymphocyte responses. "Stimulating cell type" showed significance ($F = 4.321$ and $p = 0.04$). The mean SI produced by stimulation with unrelated male control cells (M = 14.629 ± S.E.M = 1.997) was significantly higher than that produced by male spouse cells (9.553 ± 1.587). However, the difference between spouses and controls as stimulators was only evident in the non-pregnant group ($F = 8.721$ and $p = 0.003$) indicating that females in this group responded greater to unrelated male controls than to their spouses. Normal and ectopic pregnant females showed similar responses to their spouses and controls.

The strongest significance was obtained when "plasma type" was entered as the only variable in the model ($F = 3.541$ and $p = 0.008$). Hypothesis testing of the five types of plasma revealed that autologous plasma produced a significantly greater mean SI than any other type. This finding, however, limited the analysis, since autologous plasma could be

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further divided into three types depending on the pregnancy condition of the female donor. Therefore, a one-way analysis of variance followed the factor analysis model. The same factor model ANOVA was also performed using the %RR to represent MLR as the dependent variable. The results failed to show any significance regardless of the number of factors in the estimation model.

The one-way ANOVA produced 24 means for each SI (Table 1) and %RR representing all the possible combinations of the independent variables. Each of the 24 unique cultures was treated as an independent treatment and the one-way ANOVA along with Tukey's Honestly Significant Difference (HSD) was performed. PHA data and leukocyte phenotypes data were analyzed using the same methods.

Interpretation of results

The addition of plasma from normal pregnant females produced significant enhancement from autologous MLC ($p < 0.001$). This increase was seen when both the male spouse cells (Figure 7) and the male unrelated control cells (Figure 8) were used as stimulators. MLC's from ectopic and non-pregnant females were not enhanced by their autologous plasma. In addition, plasma from ectopic pregnant females failed to enhance MLC's from normal pregnant females. Collectively, these data point to an

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Table 1

Stimulation Index Means (and S.E.M) for each Treatment

Pregnancy condition	Stimulating cell type	Plasma type				
		None	Pooled male	Autologous	Pregnant	Ectopic pregnant
Non-pregnant	Spouse	7.43(2.63)	5.45(1.54)	3.99(1.03)	N/A[1]	6.28(0.99)
	Control	12.07(3.13)	12.79(4.29)	12.57(4.67)	N/A	18.14(6.06)
Normal	Spouse	8.40(2.00)	4.46(1.41)	33.38(16.07)	N/A	2.71(0.78)
	Control	8.51(2.13)	5.65(1.37)	45.98(18.94)	N/A	3.97(1.18)
Ectopic	Spouse	8.64(2.23)	10.94(3.02)	12.07(2.96)	2.61(0.79)	N/A[2]
	Control	11.30(2.31)	11.96(2.65)	16.36(4.38)	6.48(2.35)	N/A

[1] Plasma from normal pregnant females was not added to MLC's from non-pregnant or other pregnant females.

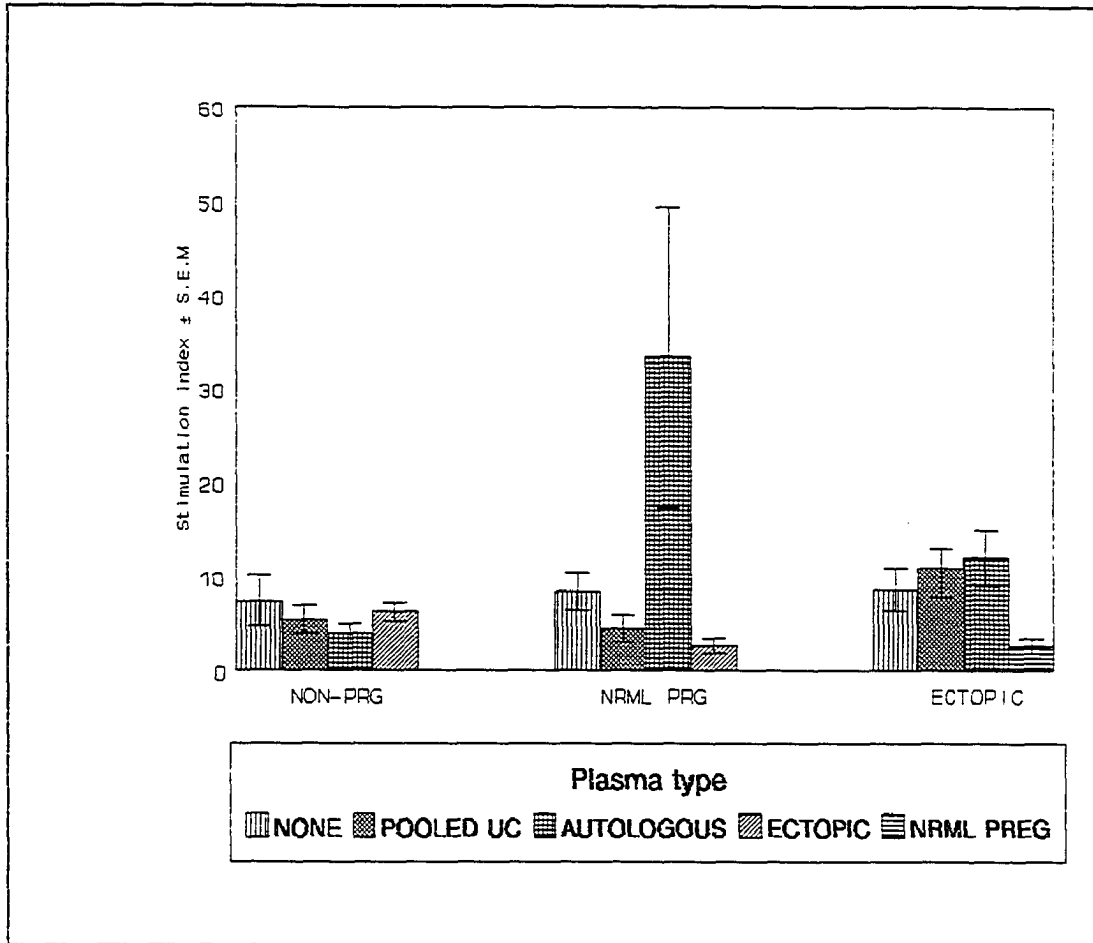[2] Plasma from ectopic pregnant females was not added to MLC's from other ectopic pregnant females.

Figure 7. Plasma effects on MLC with male spouse cells as stimulators.
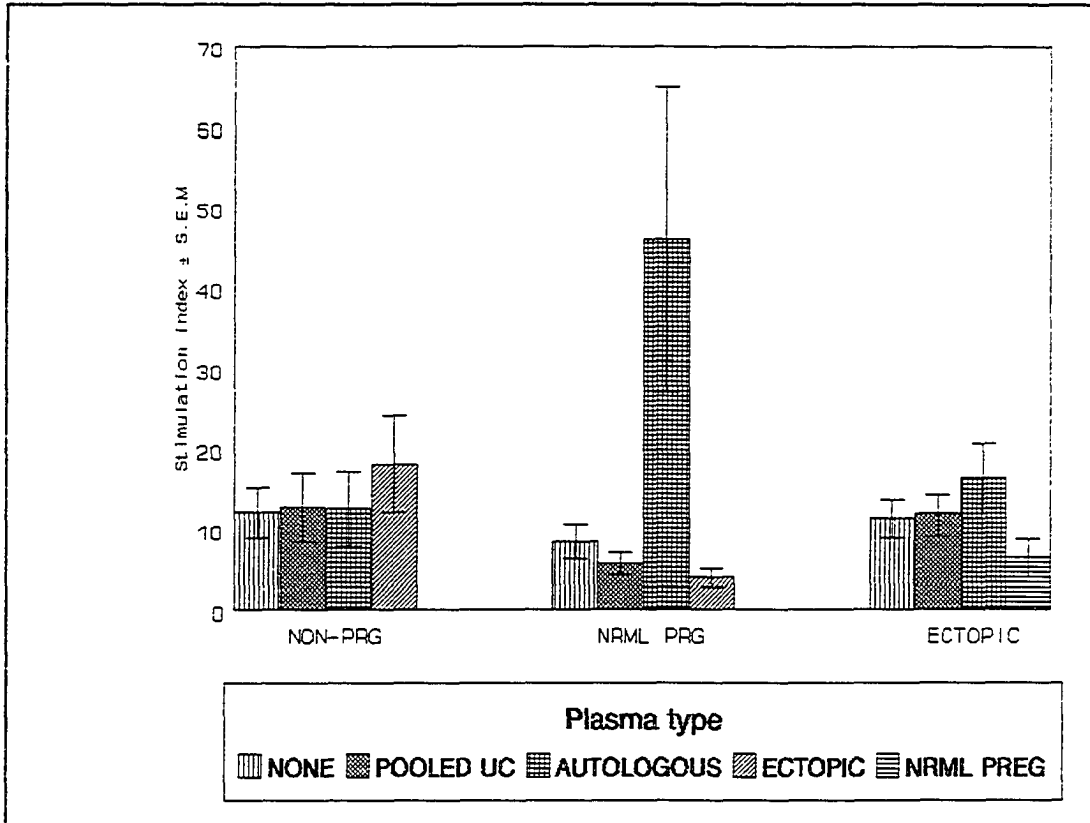
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Figure 8. Plasma effects on MLC with unrelated male control cells as stimulators.

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enhancing capability in plasma from pregnant females that is lacking in ectopic pregnant and non-pregnant females.

Moreover, enhancement was not seen when normal pregnant plasma was added to MLC's from ectopic pregnant females indicating that lymphocytes from ectopic females are incapable of responding to this enhancing factor(s). Thus, the first trimester of a normal pregnancy is characterized by two changes: a) the presence of soluble plasma factors and b) lymphocytes that are responsive to these factor(s). Both of these phenomena are lacking in ectopic gestation. Paradoxically, when plasma effects were tested on male-to-male MLC's, cultures with no plasma had a significantly higher SI than all the cultures with plasma ($p < 0.05$). Plasma showed a universal depression of stimulation regardless of its type (Figure 9).

Cultures with no plasma produced similar results in the MLC across all pregnancy conditions and with the two types of stimulator cells. The addition of pooled male plasma to all the cultures produced no significant differences among the three pregnancy conditions and plasma types.

Response rates from cultures with no plasma were used to compare the responses of females to stimulation with their male spouses relative to unrelated male controls. Response rates were also used to compare the responses of the males to stimulation with their female spouses relative to
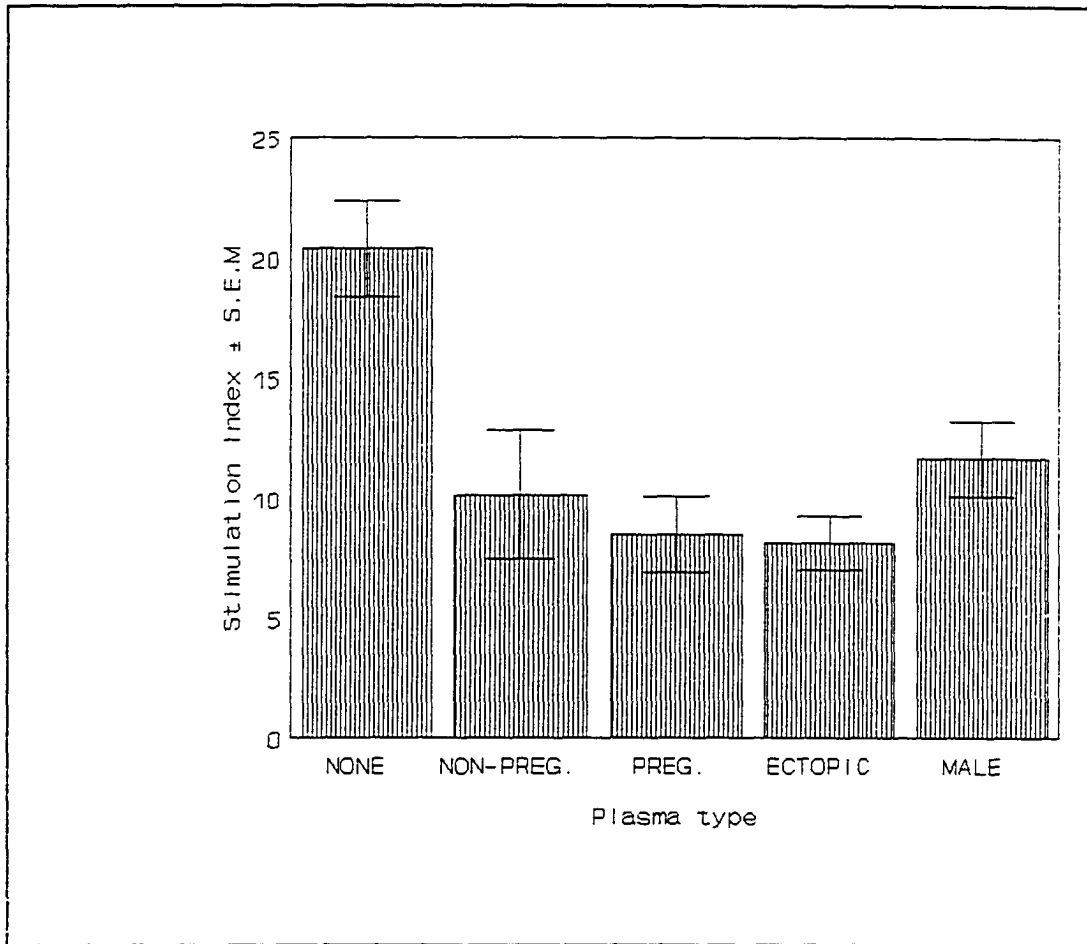
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Figure 9. Plasma effects on male mixed lymphocyte cultures.

unrelated male controls (Figure 10). With a 100% indicating an identical response of the tested group relative to controls, females showed a reduced, but not significant, response (91.092 ± 10.761) to their husbands relative to controls over all pregnancy conditions. No significant difference was encountered when response rates were compared among the three pregnancy conditions. A test of females as stimulators of their husbands lymphocytes relative to male controls also revealed no significant difference among the three conditions. None of the female lymphocytes were better at stimulating their husbands lymphocytes.

Analysis of PHA treatment revealed a significantly higher stimulation index ($F = 2.439$ and $p < 0.001$) in all cultures with no plasma. This increase was seen in all pregnancy conditions (Figure 11). There was no difference in the response of lymphocytes from the various pregnancy conditions to PHA stimulation. Both male and female cells responded in a similar fashion to PHA. As in the male-to-male MLC's, PHA cultures were significantly inhibited with the addition of any type of plasma.

The anti-paternal lymphocytotoxic assay yielded a negative reading on all test wells. Thus, the rating of "one" was assigned to each experiment and no statistical analysis was deemed necessary in lieu of the uniformity of the results. The positive and negative controls in the assay consistently performed as expected. Plasma from normal pregnant, ectopic pregnant,
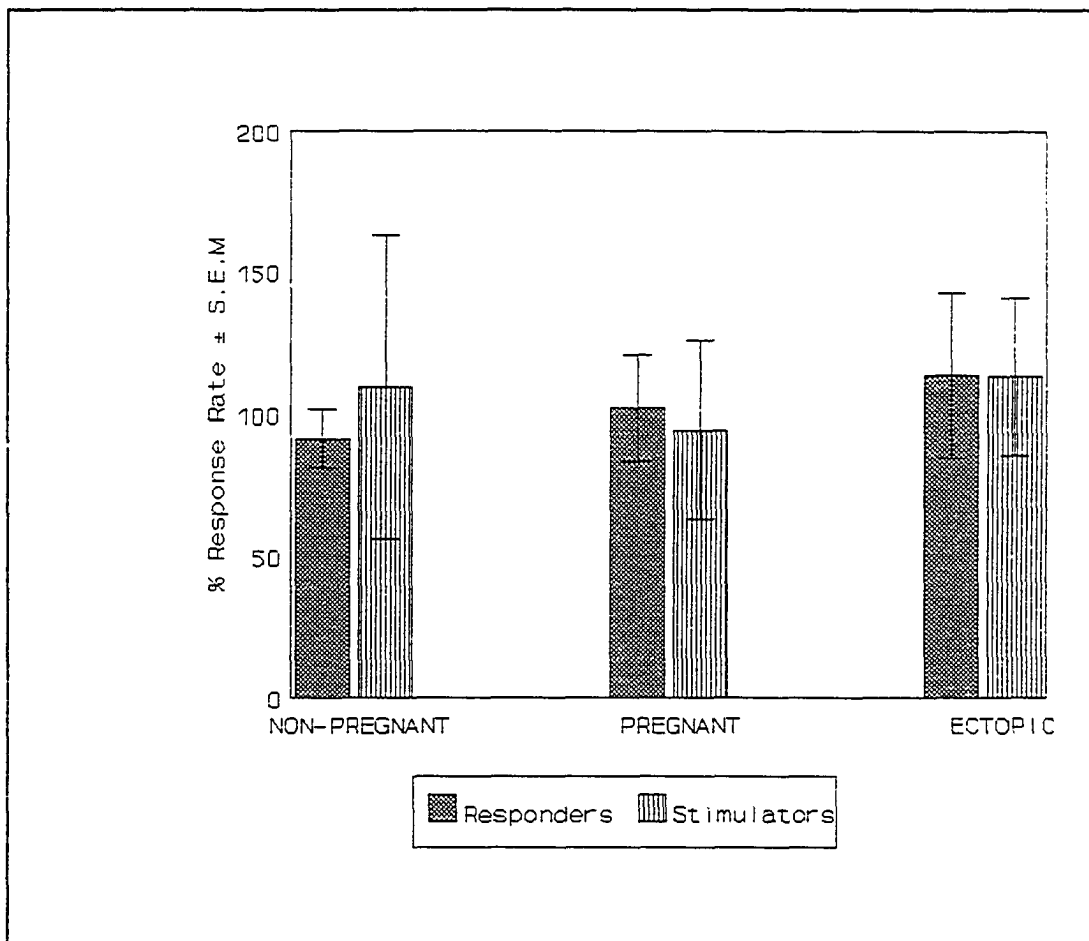
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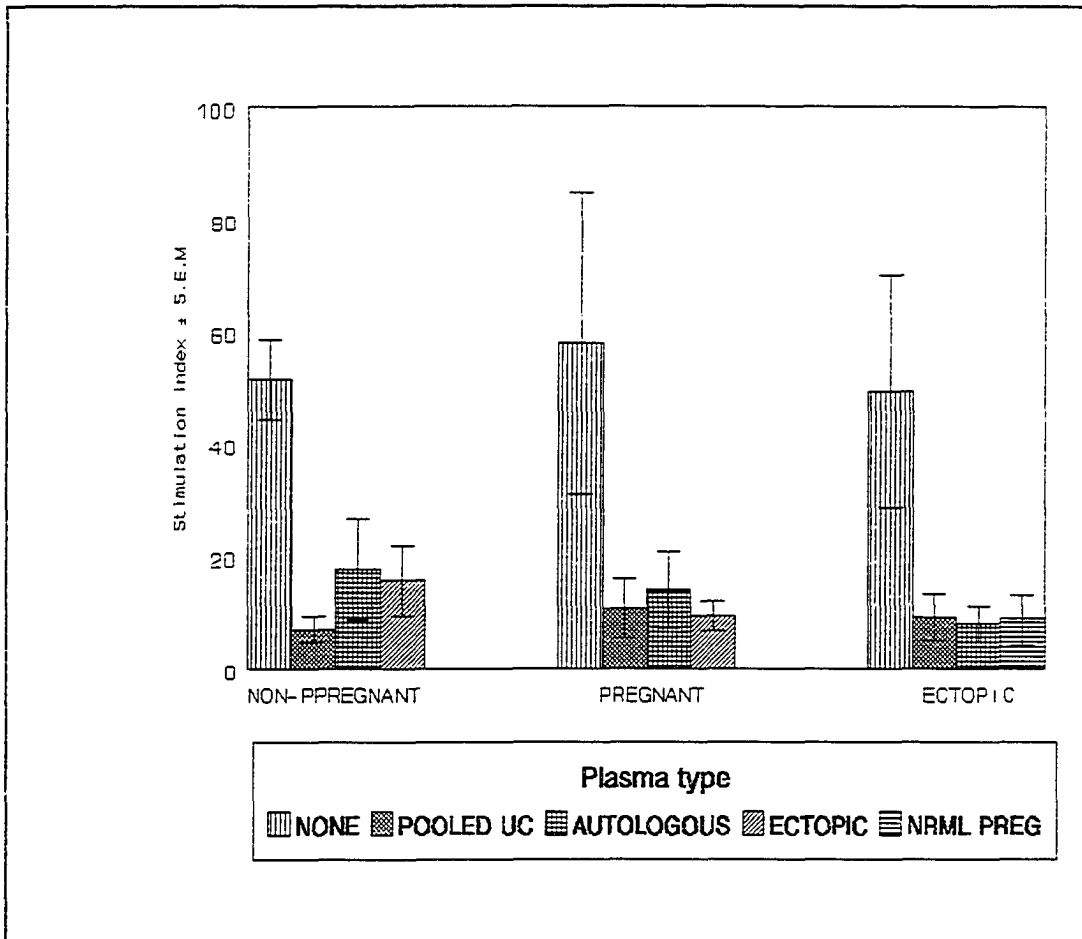Figure 10. Females cells used as responders and stimulators to their male spouses.

Figure 11. Plasma effects on PHA-stimulation.

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and non-pregnant females was marked by the absence of complement-dependent cytotoxic antibodies. This data supported the conclusion of previous studies (Regan & Braude,1987; Hasegawa, 1990) in which the presence of anti-paternal lymphocytotoxic antibodies was not always necessary for the maintenance of normal pregnancy.

The analysis of the peripheral blood leukocyte phenotypes (Figure 12) yielded no significant differences ($p > 0.05$). The percentages of circulating phenotypes were similar in the three pregnancy conditions.

Summary of results

The couples in the study were tested for immunogenetic homogeneity using MLC response rates. There was no statistical difference between the %RR among the female groups indicating that lymphocytes from all couples functioned equally well as simulators or responders. Furthermore, there were no shifts in peripheral blood leukocyte phenotypes among the three pregnancy conditions.

MLC's with male lymphocytes as stimulators were used to characterize maternal immunity within the first trimester of pregnancy . Lymphocyte proliferation of non-pregnant females to their male spouses lymphocytes was significantly lower compared to unrelated men. However, this difference was not observed during normal and ectopic pregnancy.
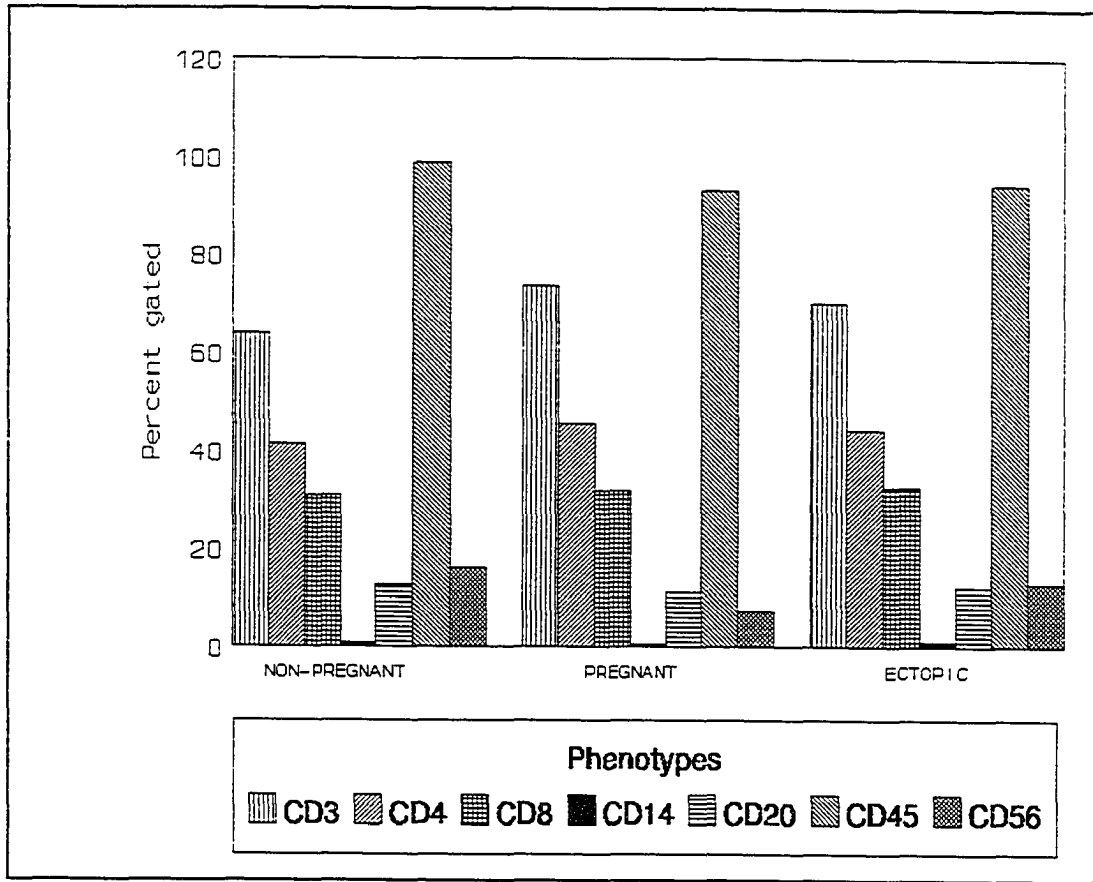
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Figure 12. Peripheral blood lymphocyte phenotype distribution.

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Female participants were also tested for immunoregulatory plasma factors. The addition of autologous plasma significantly enhanced MLC's from females with normal pregnancies when either spouse or unrelated male lymphocytes were used as stimulators. The same plasma did not enhance lymphocyte responses of females with ectopic pregnancy, suggesting that the augmentation is specific to cells from normal pregnant females. In contrast plasma from males, ectopic females and non-pregnant females did not contain any enhancing factors. Unrelated male-to-male MLC's were significantly inhibited by all types of plasma.

Female participants were also tested for the effects of plasma factors on PHA-stimulated cultures. All lymphocyte cultures proliferated over the baseline culture with the addition of the mitogen. However, there were no differences among the three pregnancy conditions. The addition of plasma regardless of source produced significant inhibition of PHA responses. The general inhibition seen in PHA-stimulated cultures supports previous findings that implicated plasma $\propto 1$- Anti-trypsin (Breit, 1982). Thus, the enhancing capabilities of plasma from normal pregnant females was restricted to allogeneic stimulation.

CHAPTER FIVE

Summary, Discussion, and Conclusions

The results of the MLC assay without the addition of plasma suggested that there are no differences in the responses of females to their spouses as compared to other male controls among the three pregnancy conditions. Thus, HLA differences or similarities between mating couples as measured by the MLC were not a predeterminant of either ectopic or normal pregnancy. The results did not indicate an immunogenetic predisposition to ectopic gestation.

Plasma from normal pregnant females has factor(s) that enhanced the response of autologous lymphocytes. This factor(s) was lacking in the plasma of ectopic pregnant and non-pregnant females.

Moreover, there was a cellular component to the increased response encountered in the cultures of pregnant women. Plasma augmentation of lymphocyte responses from normal pregnant females cannot be attributed to the plasma type alone. If the factor(s) found in plasma of pregnant women is a non-specific source of enhancement, then it is expected that all MLC's will respond to the addition of plasma from normal pregnant women. However, this was not the case. The addition of plasma from normal pregnant females did not enhance the MLC's of ectopic females and male

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MLC's. Therefore, there are cellular changes that must accompany the soluble (plasma) attributes in order to stimulate lymphocyte responses. Hence, lymphocytes from pregnant females contain a specific mechanism that is capable of responding to this factor(s) in the pregnant plasma. The mechanism of enhancement may be in the form of cell membrane receptors or other forms of cellular recognition that bring about the increased lymphocytes responsiveness.

Mixed lymphocyte responses from ectopic pregnant women exhibited unique characteristics distinguishable from normal pregnant females. Unlike MLC's from pregnant women, lymphocytes from ectopic pregnant females were not responsive to the addition of autologous or normal pregnant plasma. Even though gestational age and parity of the normal and ectopic pregnant females were comparable, lymphocytes from normal pregnancy seem to respond vigorously in the presence of their own (autologous) plasma. Plasma from ectopic pregnant females lack the enhancing soluble factor(s) present in the plasma of normal pregnant females and their lymphocytes lack the cellular elements necessary for responding to this factor(s).

Further support for the involvement of a cellular component in the observed enhancement is that the same plasma types did not exhibit similar effects on unrelated male mixed lymphocyte cultures. On the contrary, the

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addition of any plasma, regardless of type, showed a general inhibitory effect. The lack of enhancement in male-to-male MLC's point to the presence of a specific mechanism by which lymphocytes from normal pregnant females respond to the putative soluble factor(s).

Allogeneic stimulation in MLC is a product of difference in the class II HLA between maternal and paternal lymphocytes. The addition of plasma form pregnant females enhances these allogeneic responses. What, then, is responsible for the enhancement of MLR that is seen in the MLC's of normal pregnant females but not in ectopic or non-pregnant females? One of the major differences between normal pregnancy and ectopic pregnancy is the secretory dynamics of some pregnancy hormones. Progesterone levels are lower in ectopic pregnancy than in normal pregnancy (Stovall et al., 1989). Progesterone is known to up-regulate estrogen receptors (Sitteri, Febres, Clemens, Chong, Gondes, & Stites, 1977; Clemens, Sitteri, & Stites, 1979), and these receptors have been found on T-lymphocytes (Paavonen, Anderson, & Adlercreuts, 1981; Feigen, Fraser, Peterson, & Dandlikes, 1978). Therefore, in normal pregnancy progesterone may up-regulate estrogen receptors on T-cells, and the addition of autologous plasma rich with estrogen may result in T-cell proliferation through the direct action of estrogen. Estrogen is also known to inhibit suppressor activity thus adding more to hyperactivity of T-cells. Since ectopic pregnancy has lower levels

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of both progesterone and estrogen then this interaction between the immune and endocrine system is lacking and T-cell enhancement may not proceed as efficiently. Furthermore, male lymphocytes are not under the effect of progesterone and may not have the estrogen receptors. Hence, male lymphocytes may not be responsive to the addition of estrogen that is found in the plasma of normal pregnant females. Accordingly, the proposed model may explain the lack of male responsiveness to plasma from pregnant females that was demonstrated in this study.

Kasakura (1971) showed that fetal plasma as well as pregnant plasma are inhibitory to mother-child MLC, while unrelated MLC were inhibited by pregnancy plasma only. This work along with Bissenden et. al. (1980) referred to an inhibiting factor or antibody that may be produced early in pregnancy. Most subjects in these earlier studies were greater than 12 weeks gestational age. This evaluation of immune function in ectopic pregnancy presented data regarding the behavior of pregnant plasma early in the first trimester. Prior studies report a significant increase in inhibition as pregnancy progresses (McIntyre et al., 1979a; 1979b; Bissenden et al., 1980; Herva et al., 1977). The results of immunologic data in ectopic pregnancy presented in this paper along with the findings from previous research indicate that at early stages of pregnancy there is an increase in immune recognition that is then down-regulated as pregnancy progresses.

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This paper showed that early normal pregnancy is characterized by hyperactive allogeneic lymphocyte responses that are subsequently down regulated. The dynamic progression of maternal immunity from hyper- to hypo-activity and the balance between enhancement and inhibition seem to be important in successful pregnancy. This proposed balanced seems to be regulated by soluble plasma factor(s) and cellular modifications in effector cell populations. The production of irCG in MLC may hold one explanation for this bimodal effect. At low levels irCG is capable of enhancing MLR, but as its concentration increases, it shows a suppressive effect on MLR (Harbour-McMenamin, Smith, & Blalock, 1986). Pregnancy can be thought of as one-way MLC where the lymphocytes of the mother are responding to stimulation by fetal tissue. If the production of irCG and its effects on maternal lymphocytes *in vivo* are similar to those *in vitro*, then it is possible that the effects of irCG *in vivo* promote enhancement in early pregnancy and later suppression of immune responses. As a result, the immunology of pregnancy fluctuates with the progression of gestation. At early stages of pregnancy, allogeneic differences support recognition of the fetus. Recognition is needed for the onset of specific inhibitory effects such as the humoral production of blocking antibodies which will later prevent the rejection and subsequent destruction of the implanted fetus.

The model for studying feto-maternal immunologic interactions in this study selected paternal lymphocytes to represent fetal tissue and proceeded to investigate maternal immune reactions to these paternal antigens. This *in vitro* model may be used as a practical venue to study *in vivo* maternal immune responses to the implanted fetus since it may be presumed that maternal immune response to paternal antigens proximate responses produced by fetal antigens. First, the fetus can be regarded as a semi-allograft since it expresses inherited paternal antigens not found in the mother. Second, there is evidence that maternal immune reactions to fetal tissue produce humoral and cellular specificities similar to those found on paternal tissue (Horini, & Terasaki, 1982; Reed, Bonagura, Kung, King, & Suciu-Foca, 1983). Both these observations render sufficient support for the use of paternal tissue in place of fetal tissue to study feto-maternal immunobiology.

The uterus undergoes various changes in normal pregnancy that may be responsible for enhancing allogeneic activity in the first trimester. Based on data presented in this study, a probable mechanism would involve the appearance of plasma factor(s) and appropriate membrane receptors on lymphocytes. In ectopic pregnancy, uterine changes occur proximal to the implantation site, and these changes are incomplete in comparison to normal pregnancy (Cunningham et al., 1989). The retardation of normal uterine

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development in ectopic gestation may compromise its putative immuno-regulatory role and reduce alloreactivity. Moreover, in ectopic gestation the development of the blastocyst is impaired and it may present different antigens than normal trophoblast. Studies cited in the review of the literature emphasize the major role HLA play in the regulation of pregnancy. If the expression of HLA is altered due to the compromised development of the ectopic blastocyst, then the ensuing MLC studies may provide results distinguishable from normal pregnancy MLC's.

Lymphocyte studies in the peripheral blood suggest that most of the effector cell activity proximal to the uterus is secondary to the success of implantation. Earl et al. (1987) and Maruyama et al. (1992) report differences in the effector cell population between the decidua of ectopic and normal pregnant females. The presence of these unique cell populations at the site of ectopic implantation and the signals that pass between these cells and the trophoblast may explain some of the immunoregulatory differences between normal and ectopic pregnancy noted in this study. Further studies regarding the interaction between effector cell populations and fetal elements may help characterize the role the immune system plays in maintaining a successful pregnancy.

Further studies may reinforce the results of the current study by adding steps to the design that solidify the conclusions regarding the effect

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of pregnant plasma. The inclusion of a culture in which normal pregnant plasma is added to non-pregnant mixed lymphocyte cultures will help in ascertaining whether the enhancement is due to acquired cellular responsiveness in normal pregnancy and whether lymphocytes from ectopic pregnancy mimic lymphocytes from non-pregnant females in their nonresponsiveness to plasma from pregnant females. A more extensive characterization of pregnancy hormones and their effect on the immune response in early pregnancy may verify some of the proposed interactions between the immune and endocrine systems. Additional information on the production of irCG may elucidate its role in the survival of the fetus. In general, future research is needed to characterize the factor(s) presumed to be at work in normal pregnant plasma. The interaction of the various types of effector cells at the feto-maternal interface must be further defined. Moreover, there still remains the role of cytokines in maternal immunomodification. With the advent of new technology it is becoming easier to master the study of cytokine molecules, their complex effects on pregnancy, and their interactions with the endocrine system. All the previous proposed issues will provide a greater understanding of reproductive biology and how its application may aid in the control of reproductive functions and outcomes.

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In conclusion, results from this study indicated that there is an enhancement of responses in the lymphocyte responses in pregnant women when complemented with their (autologous) plasma. This phenomena was not seen in MLC's from ectopic pregnant females and may be due to the presence of plasma factor(s) in normal pregnant females in the first trimester that augments lymphocyte proliferation. In addition, there is evidence that lymphocytes from ectopic females are incapable of responding to this factor(s) in the same manner as lymphocytes from pregnant females. The study indicated that maternal immunologic responses are altered in ectopic pregnancy. This may be directly related to alterations in feto-maternal interactions and fetal development in the uterine versus tubal environment. Further characterization of these findings may provide greater knowledge regarding the immunologic role of the intrauterine environment in supporting normal pregnancies.

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APPENDIX A

Determination of Cell Concentrations

Objective

This experiment was performed to determine the ratio of responder to stimulator cells to be used in each MLC assay.

Procedure

Lymphocytes were separated, cultured, and treated with Mitomycin-C in accordance with the protocol outlined in chapter three. A matrix setup outlined below included three responder and stimulator cell concentrations. The stimulation index (SI) was calculated using the formula listed in chapter three. "R" represented the responding lymphocyte population, "Rx" represented the stimulator population in the autologous culture. Two unrelated controls (UC1x, UC2x) were also used as stimulators in the mixed lymphocyte cultures.

Result

There was no significant difference in the SI obtained using 5×10^4 or 10×10^4 stimulator-to-responder cell combinations per well. Using 20×10^4 for either the responder or stimulator was not feasible due to the large amount of blood that would be needed from each participant (Table B1). Therefore, the concentration used in this study was: 5×10^4 responder cells and 5×10^4 stimulator cells per well.

Table B1

Stimulation indices of various combinations of stimulator and responder cell

concentrations per well

	Responder cells per well		
	$R\ 5 \times 10^4$	$R\ 10 \times 10^4$	$R\ 20 \times 10^4$
Stimulator cell per well			
UC1x 5×10^4	19.91	19.83	18.25
UC1x 10×10^4	11.59	18.51	13.28
UC1x 20×10^4	80.62	47.34	37.08
UC2x 5×10^4	47.78	32.16	16.89
UC2x 10×10^4	24.26	40.30	30.22
UC2x 20×10^4	99.83	73.69	51.90

Determination of Mitogen Concentration

<u>Objective</u>

The objective of the experiment was to determine whether 1:100 or 1:200 dilution of PHA is more effective for maximum lymphocyte proliferation.

<u>Procedure</u>

Lymphocytes were separated, cultured, and treated with Mitomycin-C in accordance with the protocol outlined in Chapter three. A matrix setup outlined below included two concentrations of PHA. All the wells contained 5×10^4 cells. "R" represented the responding cell population, "Rx" the Mitomycin-treated cell population. The results were expressed as SI's and were calculated according to the formula listed in chapter three.

<u>Result</u>

The results indicated that a 1:100 dilution was optimal for the growth and proliferation of lymphocytes in the culture media (Table B2).

Table B2

Stimulation indices of two PHA concentrations

	R	$PHA_{1:100}$	$PHA_{1:200}$
Rx	NA	0.50	0.54
$PHA_{1:100}$	25.38	NA	NA
$PHA_{1:200}$	7.30	NA	NA

MIXED LYMPHOCYTE CULTURE REPORT

Patient I.D. (R):	000000000000C			DATE:	
Spouse I.D. (S):	000000000000C			FILE:	
Unrelated Control 1 (UC1):	000000000000C			CLASS.	
Unrelated Control 2 (UC2):	000000000000C				

		R	S	UC1	UC2	PHA
Rx						
	cpm	3436.00	7826.00	5016.00	7777.00	774.00
	%RR	0.44	129.69			XXXXXXXXX
	SI	1.00	1.96	25.99	18.92	ERR
Sx						
	cpm	6349.00	3986.00	3102.00	6580.00	1191.00
	%RR	53.75	0.38			XXXXXXXXX
	SI	1.85	1.00	16.07	16.01	ERR
UC1x						
	cpm	6983.00	7178.00	193.00	4109.00	1274.00
	%RR	0.21	0.21			XXXXXXXXX
	SI	2.03	1.80	1.00	10.00	ERR
UC2x						
	cpm	10729.00	6716.00	8278.00	411.00	922.00
	%RR	0.14	0.22			XXXXXXXXX
	SI	3.12	1.68	42.89	1.00	ERR
PHA						
	cpm	2657.00	7584.00	6567.00	4741.00	XXXXXXXXX
	%RR	0.58	0.19	XXXXXXXXX	XXXXXXXXX	XXXXXXXXX
	SI	3.09	6.28	5.20	2.33	XXXXXXXXX
MEDIA						
	cpm	860.00	1208.00	1262.00	2037.00	XXXXXXXXX
	%RR					
	SI	1.00	1.00	1.00	1.00	XXXXXXXXX

Subscript (x) indicates Mitomycin−C−treated cell populations.

SI= Experimental MLC / Autologus MLC (RSx/RRx,...,etc).

Autobiographical Statement

Tawfiq A. Nasser was born in Jerusalem on August, 19, 1964. He came to the United States in 1983. He received a Bachelors of Science in Medical Technology at Old Dominion University on May 1987. He then joined the staff of the Delmarva Migrant Health Project on the Eastern Shore of Virginia were he served as the clinical laboratory supervisor. During that time he received his certification as a Medical Technologist by the American Society of Clinical Pathologists. He returned to Old Dominion University for a Master's Degree in Medical Laboratory Sciences. As a graduate student he worked in the College of Health Sciences, Clinical Practice Center as a research assistant and was promoted to become the Director of the Center. He also taught laboratory courses in Medical Parasitology and Hematology at the School of Medical Laboratory Sciences and Environmental Health. He received his Master's of Science degree in Medical Laboratory Sciences in August of 1989.

He left the Clinical Practice Center to work as a Clinical Associate in the Clinical Chemistry Reference Laboratory of Sentara Norfolk General Hospital. He continued to earn his Doctorate of Philosophy in Urban Health Services with a co-major in Biomedical Sciences. His research area was in Reproductive Immunology.

Tawfiq Nasser is an Associate Member of the American Society of Clinical Pathologists, he is a member of the Phi Kappa Phi Honor Society and the Alpha Eta Honor Society for the Health Sciences.