

Reproductive Immunology: Biomarkers of Compromised Pregnancies

by W. Page Faulk,* Carolyn B. Coulam,* and John A. McIntyre*

The objective of this paper is to consider several categories of biomarkers of human pregnancy. The design of the report is to discuss useful and promising markers and techniques. Research gaps, needs, and priorities are also defined. Useful markers are mixed lymphocyte culture reactions, measures of lymphocytotoxic antibodies, histocompatibility (HLA) typing, and immunohematological evaluations. Promising markers are measures of major basic protein and early pregnancy factor, as well as determinations of trophoblast-lymphocyte cross-reactive (TLX) antigens. Promising techniques are fluorescence-activated cell-sorter analysis of maternal blood for fetal and extraembryonic tissues and immunotherapy with TLX and other antigens to prevent spontaneous abortion. It is concluded that immunology has much to offer the development of biomarkers of human pregnancy.

Introduction

Efforts to understand the immunology of human pregnancy have focused on extraembryonic membranes. The rationale for this approach is the point of contact between maternal tissues and the conceptus is trophoblast. Cells of the inner cell mass differentiate into the embryo, while extraembryonic components form an interface with maternal blood and uterine cells (1). This materno-trophoblastic interface exists at all anatomic sites in contact, including placenta, amniochorion, spiral arteries, basal plate, and interstitial tissues.

Because of its location, the trophoblast is important in potential maternal immune recognition and rejection reactions. Its plasma membranes are unique inasmuch as none of them express the polymorphic form of class I or class II transplantation HLA (histocompatibility) antigens; however, certain cytotrophoblast are reactive with monoclonal antibodies thought to recognize monomorphic (i.e., class I HLA framework) antigens. This lack of transplantation antigens has led to speculation about trophoblastic immunological neutrality; however, numerous investigators have shown trophoblast membranes are not immunologically inert (2).

Antibody Aspects of Trophoblast Immunogenicity

The immunogens which signal and maintain maternal recognition are extraembryonic structures at the materno-trophoblastic interface called trophoblast anti-

gens (3). Evidence to support this comes from studies of cell-mediated immunity which show certain trophoblast antigens (4) and some antitrophoblast antibodies (5) can modulate allogeneic recognition reactions. Although antitrophoblast antibodies have been identified in some normal and abnormal pregnancies (6), such activity cannot be identified in most normal pregnancy sera. For example, the enzyme-linked immunosorbent assay (ELISA) identifies antihuman trophoblast antibodies more readily early than late in a first pregnancy, and more efficiently in first than in subsequent pregnancies (7).

There are at least four explanations why antitrophoblast antibodies are not identified in all pregnancy sera: antibody combining sites may be bound by trophoblast antigens in immune complexes; trophoblast immunogens may stimulate the production of blocking or incomplete antibodies; auto-anti-idiotypic antibodies within the network hypothesis of antibody control may disallow the demonstration of antitrophoblast immunity; or inhibitors of antigen-antibody reactions or of the manifestations thereof (such as complement fixation) could mask the presence of antibody. There is evidence to support all of these possibilities; a brief discussion of each follows.

Immune Complexes

Immune complexes have been suggested as a factor in the decreasing ability to demonstrate antitrophoblast antibodies (8). This is supported by experiments that show increased circulating immune complexes during pregnancy, although these results are controversial.

*Methodist Center for Reproduction and Transplantation Immunology, Methodist Hospital of Indiana, Indianapolis, IN 46202.

Studies of human pregnancy sera have revealed the presence of circulating immune complexes composed of five biochemically identifiable trophoblast-antitrophoblast components, two of which can also be identified in nulliparous nonpregnant female sera.

Increased quantities of trophoblast antigens have been reported in maternal sera as pregnancy progresses (1). This has been cited as further support for trophoblast antigen-containing immune complexes. Such complexes could account for the difficulty in demonstrating antitrophoblast immunity in some maternal sera.

Blocking or Incomplete Antibodies

Blocking and/or incomplete antibodies have long been suspected as being important manifestations of immunity in reproduction and cancer research. Techniques were developed many years ago to identify antibodies that were not serologically detectable by conventional assays (9). These methods have been used to demonstrate otherwise undetectable antibodies in animals rendered tolerant as neonates to allogeneic cells (10). Such blocking or incomplete antibodies have been described in host responses to transplants, cancers, tolerance induction, and pregnancy.

Maternal antipaternal-blocking immunity appears to be important in normal pregnancy, for such immunity is absent in certain abnormal pregnancies (6,11). That trophoblast may initiate blocking responses during pregnancy is supported by experiments in mice that show that placental extracts presented during the onset of immunization with an unrelated antigen promote the production of blocking, and do not promote cytotoxic antibodies (12). Blocking antibodies could be a cause of the failure to consistently demonstrate antitrophoblast immunity in maternal blood.

Auto-anti-idiotypic Antibodies

Auto-anti-idiotypic antibodies to anti-HLA antibodies in recipients of donor-specific transfusions have been found in sera of patients who lack demonstrable serological responses to HLA, indicating that the presence or absence of detectable antibody may be a reflection of the amount of anti-idiotypic (13). Sera from persons alloimmunized through pregnancy, transfusion, or transplantation can react with autologous T-lymphoblasts primed against the immunizing donor (14).

These observations coupled with the finding that primed T-cells display idiotypelike receptors for alloantigens has prompted an idea that T-cell receptors induce the formation of anti-idiotypic antibodies. Such auto-anti-idiotypic antibodies to HLA are a general finding during and after pregnancy (15). These antibodies may help to understand why antitrophoblast antibodies cannot be demonstrated in all maternal sera.

Inhibitors of Antigen-Antibody Reactions

Inhibitors of antigen-antibody reactions have been described in both complement-dependent (16) and com-

plement-independent (17) assays. Complement-dependent cytotoxicity of paternal lymphocytes by maternal antibodies to trophoblast antigens is impeded if the serum either is heated 30 min at 56°C or absorbed with immobilized heparin. The inhibitor can be removed by a wash step before the addition of complement, and cytotoxicity proceeds normally.

The inhibitor of complement-independent hemagglutination reactions has been shown to bind concavalin-A. It is activated by heating at 56°C for 30 min and is under the control of an inhibitor-of-inhibitor, which is present in plasma but not serum (17). The inhibitor-of-inhibitor is heat labile, sensitive to calcium concentrations, destroyed by Russell's viper venom, and absent from the plasma of patients with a deficiency of clotting Factor V, suggesting that the blood clotting system may play a role in modulating maternal antibody-antigen interactions within the placental bed.

Knowledge about the inhibitors and inhibitors-of-inhibitors of antigen-antibody reactions is only beginning to emerge; they could be important in some failures to demonstrate maternal antitrophoblast antibodies, particularly if the sample is heated or if no attention has been given to the type of anticoagulant used or to the calcium concentration.

Useful Immunobiomarkers in Human Pregnancy

In normal pregnancy, B-lymphocyte activation by trophoblast antigens can be demonstrated by the elution of maternal antitrophoblast antibodies from homogenates of individual placentae (18). T-lymphocyte activation by trophoblast can be shown by demonstrating that chemically defined trophoblast antigens (19) reversibly block T-lymphocyte responses to B-lymphocytes in mixed lymphocyte culture (MLC) reactions (4). Normal maternal pregnancy plasma causes a similar blockade of maternal T-cells to respond to paternal B-cells in MLC reactions (20). Certain laboratory tests of maternal immune responses to pregnancy have proven to be clinically useful in the diagnosis and management of high risk pregnancies. Four of these are considered below.

Mixed Lymphocyte Culture (MLC) Reactions

One of the best examples of cell-mediated immunity in pregnancy is the MLC reaction. In this reaction, lymphocytes from two different individuals are mixed together and cultured under conditions that permit measurement of their DNA metabolism, which is an index of the intensity one cell reacts to the other. As a model of pregnancy, the father's cells are cultured with the mother's cells. The father's cells are sufficiently irradiated to disallow their ability to immunologically respond, but their capacity to stimulate the mother's lymphocytes is retained. This is called a one-way MLC reaction.

One-way MLC reactions between a pregnant woman and the father proceed normally in the mother's non-pregnant or third-party serum or plasma, but are blocked by plasma from the pregnant mother (20). The most widely accepted explanation for this blockage of allogeneic recognition is that it is caused by blocking antibodies in the pregnant mother's blood. These antibodies have specificities for allotypic trophoblast antigens on the father's lymphocytes.

Convincing support of a role for blocking factors in pregnancy comes from clinical investigations of unexplained spontaneous abortions. Many women with primary recurrent spontaneous abortions do not produce a factor in their blood that blocks *in vitro* models of cell-mediated immunity between lymphocytes from the mating partners (21). In some cases, this deficiency can be overcome by immunizing the woman with lymphocytes (22).

Lymphocytotoxic Antibodies

Lymphocytotoxins in pregnancy sera have been traditionally interpreted as being anti-HLA (23). This is clearly not the case in secondary spontaneous abortion, for cytotoxicity is removed from sera by absorption with HLA-negative trophoblast or HLA-unrelated platelets (24). Some pregnant patients produce non-HLA lymphocytotoxins, and results from experiments of idiotype-anti-idiotypic immunity during pregnancy indicate that broadly reactive lymphocytotoxins with TLX specificity are not uncommon in normal pregnancy (24). These findings are compatible with the idea that normal pregnancy requires maternal immunological recognition of the trophoblast antigens (3). Absent or inappropriate recognition results in failed or faulty implantation of the blastocyst (1).

There is much speculation about the nature of the immunogen associated with normal pregnancy. Results of animal model studies have prompted some investigators to suggest that maternal recognition is dependent upon incompatibility of major histocompatibility complex (MHC)-encoded antigens (25). This is supported by the success of outbred as opposed to inbred matings, the benefit of allogeneic third-party leukocyte immunizations on primary spontaneous abortion (26), and by research showing a beneficial effect on pregnancy outcome in mice when the female is mated with an MHC-incompatible male (27).

It is a matter for research to determine the qualitative and quantitative parameters of lymphocytotoxins. From a practical point of view, lymphocytotoxic antibodies are markers of secondary spontaneous abortion. Diagnostic characteristics of these antibodies are broad (i.e., non-HLA) specificity; presence during nonpregnancy; high titer (i.e., higher than normally found in the same assay for anti-HLA); removal by pooled trophoblast absorption; removal by absorption with paternal platelets; and loss of reactivity following heating or absorption with solid-phase heparin (16).

Histocompatibility (HLA) Typing

The role of MHC antigens as predictors of successful pregnancy outcome has been controversial (28). Reports have supported (29,30) and refuted (31,32) an association between HLA and reproductive performance. The variation in results can be explained by the small sample sizes and the lack of homogeneity of the populations investigated. Controversy involving association of HLA and reproductive performance can be explained by properly classifying recurrent spontaneous aborters and unexplained infertility.

Infertility is classified as primary or secondary. Primary infertility designates those couples who never have conceived, and secondary infertility designates couples who have conceived but have failed to deliver during one or more years of unprotected intercourse (33). Primary infertiles have a lower theoretical probability of producing heterozygote offspring, and secondary infertiles have more HLA-B antigen homozygosity (34). Primary aborters have more HLA sharing between spouses than do either secondary aborters or childbearing controls (26). HLA typing is a common and widely available test, and can be used as a reliable laboratory procedure to assist in the differential diagnosis of spontaneous abortion.

Immunity, Clotting, and Pregnancy Wastage

Spontaneous abortion is common in patients with certain autoimmune diseases (35). Lupus patients can have lymphocytotoxins (36), lupus anticoagulants (37), or anticardiolipin antibodies (38), all of which are associated with pregnancy wastage (39). These antibodies may interrupt pregnancies because some of them react with trophoblast antigens (40). Lupus anticoagulant and anticardiolipin are antiphospholipid antibodies that react with platelets and endothelium with the net effect of prolonging laboratory measures of clotting time and promoting *in vivo* thrombosis (41) (Fig. 1).

The most widely used clinical test to measure antibodies that interfere with blood clotting is the activated partial thromboplastin time (42). In the future this will be replaced by more direct immunoassays for antibodies to phospholipids (43). Indeed, antibodies to cardiolipin are usually measured by immunoassay (44). It has been proposed that lupus anticoagulant and anticardiolipin may be the same or closely related antibodies (45). The identification of either or both antibodies in a sample of blood from a pregnant woman should signal a suspicion that the patient is at high risk of losing her pregnancy.

Promising Immunobiomarkers in Human Pregnancy

This section includes results of research on the measurement of maternal major basic protein (MBP) in the diagnosis of labor before clinical labor begins, the detection of early pregnancy factor (EPF) to determine

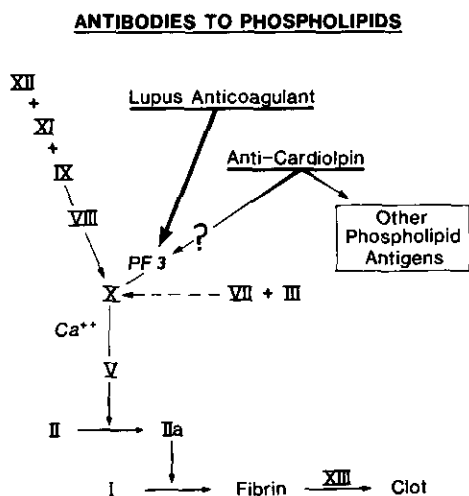


FIGURE 1. Antibodies to phospholipid components of platelet factor-3 (PF3) can interfere with assembly of prothrombinase and prolong *in vitro* measures of clotting time, such as the aPTT. These and other antiphospholipids, such as anticardiolipin, are thought to promote thrombosis by inhibition of prostacycline release from endothelium and release of thromboxane from platelets.

fertilization before implantation, and the immunobiological and immunopathological importance of trophoblast-lymphocyte cross-reactive (TLX) antigens in the assessment of high-risk pregnancies.

Major Basic Protein (MBP)

MBP forms the core of the eosinophil granule and accounts for most of the granule protein (46). It causes histamine release from mast cells and basophils (47), interacts with coagulation factors (48), and alters smooth muscle contractility (49). In human pregnancy, MBP increases in peripheral blood independent of either eosinophils or other eosinophil proteins (50), and has been shown by using immunohistological techniques to be localized in extravillous trophoblast (51). Pregnancy-associated MBP has been purified from human placenta. It is a strongly basic protein (pI > 11) with a molecular weight of 14,000 and is biochemically indistinguishable from eosinophil granule MBP.

MBP blood levels rise by 6 weeks of gestation and return to normal by 6 weeks postpartum (50) (Fig. 2). Quantitative studies indicate MBP levels plateau by 20 weeks of gestation at values more than 10 times the nonpregnant state, and they rise sharply in the third trimester in women who experience a spontaneous onset of labor. This late increase accounts for 40% of the total increase of MBP, and the increase begins at least 3 weeks prior to the onset of labor. Women who experience preterm labor have a similar increase (Fig. 3); those with oxytocin-induced labor do not, nor do those with prolonged gestation. These observations suggest that increased MBP values are markers for the onset of term or preterm labor.

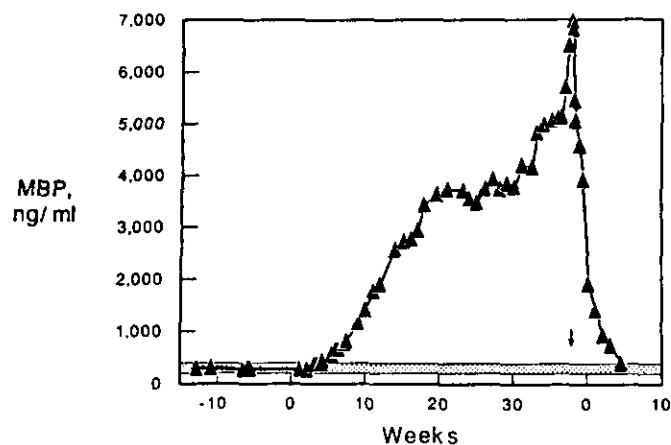


FIGURE 2. Plasma major basic protein (MBP) levels in one woman during pregnancy. From Wasmoen et al. (72).

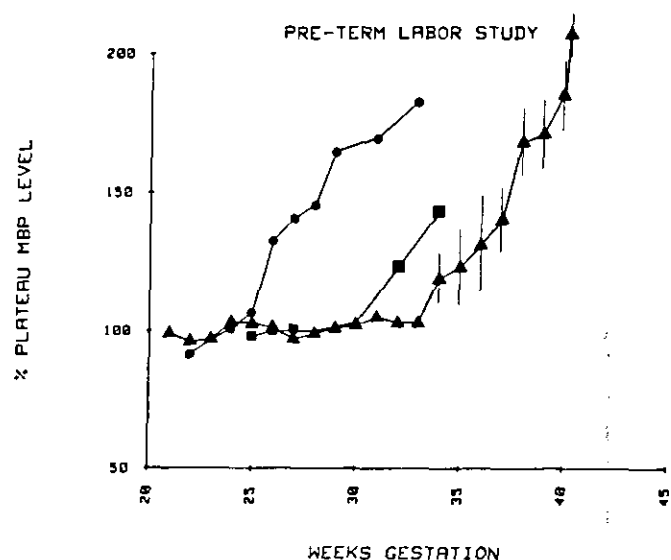


FIGURE 3. Serum major basic protein (MBP) concentrations in women with spontaneous term and preterm labor. (▲) MBP levels in six women with term labor; (●) MBP levels in a woman with spontaneous onset of preterm labor; (■) MBP levels in a woman with spontaneous rupture of membranes followed by preterm labor and delivery at 34 weeks of gestation. From Coulam et al. (73).

Early Pregnancy Factor (EPF)

EPF is an immunosuppressive molecule, which is measured by its capacity to enhance the ability of antilymphocyte antibodies to inhibit active spontaneous rosette formation between lymphocytes and red cells (52). EPF is produced first by the mother within 24 hr of fertilization and wanes in midpregnancy, by which time its function is replaced by an embryonic (placental) form of EPF. Maternal and embryonic EPF seem to be indistinguishable. Both depend on the presence of a viable embryo. In the mother, the molecule is assembled from an oviduct component (EPA-A) and an ovarian component (EPA-B), which is synthesized under the

influence of a pituitary factor (prolactin) and an ovum factor (53) (Fig. 4).

The molecule has attracted attention because it appears so early in maternal blood following implantation. It seems also to be detectable in urine (Halle Morton, personal communication, 1987). This has the practical effect of providing a method of distinguishing truly infertile couples from women who are becoming pregnant but aborting very early. EPF is reported to be present only during pregnancy when there is a viable embryo, and as such it could be used as an early marker of embryonic viability. Another point of clinical interest is that its detection in a nonpregnant patient suggests the presence of a tumor of germ cell origin (54).

Trophoblast-Lymphocyte Cross-Reactive (TLX) Antigens

Studies of large number of HLA-characterized lymphocytes with individual TLX antisera have established TLX antigens as being alloantigens (55). These investigations also have provided serological data that TLX antibodies do not detect HLA or ABO antigens. Statistical analyses of data from studies done with the use of rabbit antitrophoblast antibodies suggest there are three TLX groupings (termed TLX-1, TLX-2 and TLX-3) (56), and studies with T- and B-cell preparations indicate the allotypes are restricted to T-cells (57).

TLX antigens have been demonstrated in seminal plasma by cytotoxicity assays and by using ELISA with rabbit and/or human antibodies on seminal plasma-coated microtiter plates (24). These antibodies have been used to identify TLX allotypy within seminal plasma. Ultracentrifugation of sperm-free seminal plasma removes TLX reactivity, as though the antigen is present on a particulate structure like a cell membrane. This may be required for antigen presentation and recognition by the female recipient. Maternal recognition of TLX antigens in seminal plasma (Fig. 5) is thought to signal maternal recognition and immunological protection of the blastocyst (58).

Patients with recurrent secondary spontaneous abortions have had one or more viable pregnancies or in-

trauterine deaths with the same mate before having spontaneous abortions. Tissue typing of father and mother usually reveals no HLA antigen sharing. These women have high-titered, persistent antipaternal immunity as represented by complement-dependent and complement-independent lymphocytotoxins (59). The maternal antibodies are broadly reactive on HLA-characterized cells; they cannot be assigned individual HLA specificities. The women's sera also are nonspecifically inhibitory for MLC reactions.

Secondary recurrent spontaneous aborters are not candidates for immunotherapy with leukocytes. The immunological basis for this is that secondary aborters are not TLX-compatible with their mates, and they appear to respond inappropriately to their mates' TLX seminal plasma antigens, inasmuch as they do not mount appropriate anti-idiotypic responses (Fig. 6). Some secondary spontaneous aborters have been successfully treated with heparin injections (26). The basis for this comes from reports that the passage of secondary aborter sera through immunosorbent columns of solid-phase heparin abolishes maternal antipaternal cytotoxicity (16).

Promising Immunological Techniques for Human Pregnancy

Molecular biology, genetics, and immunology have discovered much common ground in the past several years. Pregnancy research has benefited from these discoveries, and two promising procedures will be discussed.

Fluorescence-Activated Cell-Sorting (FACS)

This method employs fluorochrome-labeled antibodies to identify and quantitate membrane markers on cells in a heterogeneous mixture (60). FACS has been used to measure the flux of fetal cells into maternal blood during pregnancy. The technique can be employed to isolate immunologically marked cells from a complex mixture such as blood. FACS has been used to measure trophoblast membranes in pregnant mothers' peripheral circulation (61). The interesting thing about this approach is the possibility of harvesting fetal cells for cytogenetic investigations without resorting to the more invasive techniques of amniocentesis or chorionic villus biopsy.

Immunotherapy to Prevent Spontaneous Abortion

Immunotherapy for the prevention of primary spontaneous abortions was begun in 1979 (29). Primary-aborting women were transfused with buffy coat-enriched plasma from nonpaternal blood donors (22). Results thus far for more than 45 couples have shown a successful pregnancy rate, comparable to that of normal

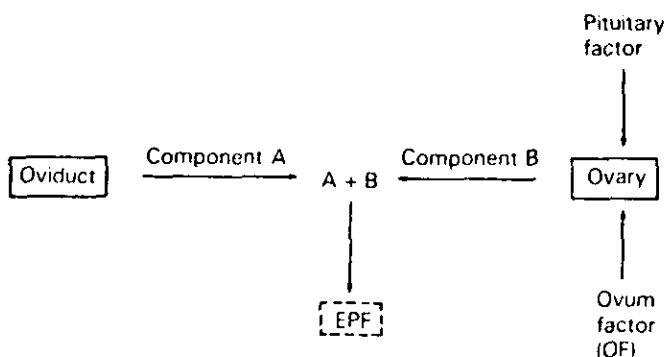


FIGURE 4. Assembly of early pregnancy factor (EPF). The pituitary factor is prolactin.

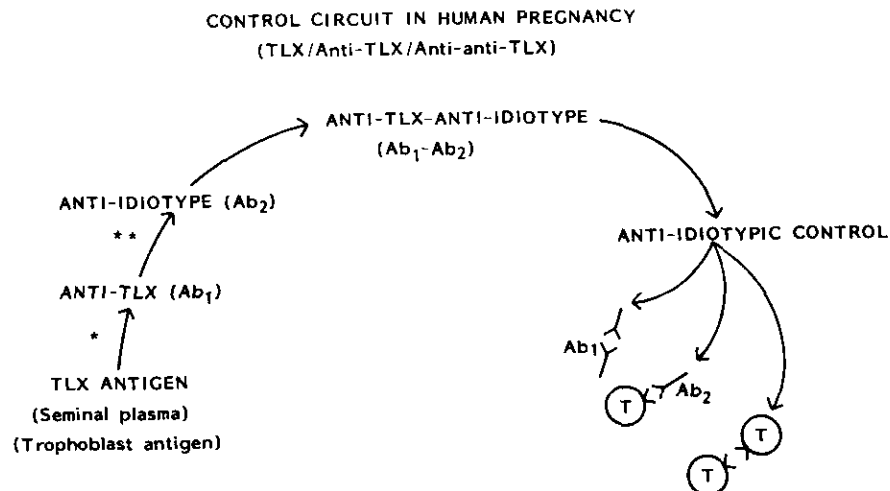


FIGURE 5. Control circuit in human pregnancy. (*) indicates site of defect manifest by recurrent primary spontaneous aborters, i.e., lack of maternal recognition of paternal TLX antigens in seminal plasma. (**) indicates site of defect manifest by recurrent secondary spontaneous aborters, i.e., maternal anti-idiotypic response to anti-TLX antibody.

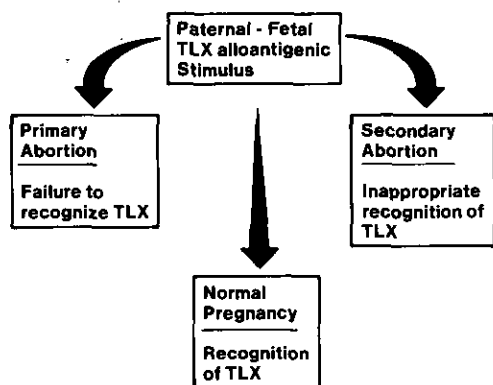


FIGURE 6. Summary of role of TLX antigens in normal and abnormal human pregnancies.

childbearing women. No graft versus host (GvH) reactions have been observed in any of the offspring (26), and no evidence of intrauterine growth retardation (IUGR) has been found. Other groups have used paternal cells in immunotherapy to prevent spontaneous abortion (30). They have observed perinatal problems such as IUGR and GvH reactions. Another group has conducted a controlled study with the use of paternal lymphocytes and found no serious perinatal problems, an absence of GvH reactions, and results comparable to those obtained with the use of nonpaternal blood donors (62).

Immunotherapy for the prevention of primary spontaneous abortion has slowed due to the danger of HTLV III in blood. Screening for HTLV III should help to alleviate some of the anxiety about such immunotherapy programs; meanwhile, several groups are studying the possibility of devising methods of TLX stimulation without using leukocytes. Techniques presently under in-

vestigation involve the use of trophoblast membranes, seminal plasma, or platelets. Double blind studies of trophoblast antigens in immunotherapy are currently underway at the University of Liverpool in England, and a controlled investigation of seminal plasma antigens is being done at the Methodist Center for Reproduction and Transplantation Immunology in Indianapolis.

Research Gaps/Needs in Pregnancy Immunobiomarkers

Measures of Spiral Artery Function

As well as can be discovered by using immunohistological techniques, the endovascular cytotrophoblast secretes a molecule found in amniotic epithelium that is designated as amnion antigen-3 (AA3) (63). This material is associated with changing spiral artery histochemistry and, presumably, function (64). Inasmuch as placental perfusion depends upon maternal blood flow through spiral arteries, it is important to develop techniques to measure spiral artery function.

Ultrasound has provided a way to determine the effectiveness of spiral artery function, but it would be more informative to develop quantitative biochemical measures of placental bed perfusion. None of the currently studied placental proteins have proven of value in this regard, but not all known placental proteins have been studied. The AA3 protein should be studied because it appears to be central in the allogeneic relationship of maternal endothelium with extraembryonic cytotrophoblast (65). Other markers should also be sought.

Developments in Immunohematology

Human placentae exist inside the uterus, but outside the body. Maternal blood has to leave the intravascular

space through the uteroplacental arteries to enter intervillous spaces of the placental bed. In this circumstance mother's blood flows past allogeneic tissues and re-enters the maternal circulation through the uterine veins. This curious pattern of maternotrophoblastic circulation during normal pregnancy is associated with quantitative changes in clotting factors and alterations in the fibrinolytic system (66).

Antibodies to phospholipids manifest as lupus anticoagulants and anticardiolipin antibodies are presently thought of as qualitative observations, but this is due to lack of precision in the assay systems and to lack of biochemical definitions of the antigens involved. With better assays and more clearly defined antigens, some of the currently used immunohematological tests will be useful biomarkers in normal and/or high risk pregnancies.

Role of Animal Models in Human Pregnancy Research

Two important general messages have emerged from research on animal models in reproductive immunology: the need for genetic diversity between mating partners, and the key role played by trophoblast in materno-fetal interactions (67). Clinical studies of the reproductive performance of women with repeated pregnancy losses and multiple partners have revealed the same two important general messages (68,69). In light of the success of preventing primary spontaneous abortions by immunizing human mothers with the father's (62) or third-party (26) leukocytes, an idea arose that pregnancy wastage in CBA \times DBA matings in mice might be prevented by a similar type of immunotherapy. Primiparous, nulliparous CBA female mice immunized with third-party leukocytes and mated to DBA/2 males do have increased pregnancy success (70,71), and a series of immunogenetic studies have been performed to explore the mechanisms of improved pregnancy outcome following immunotherapy in these animals (25).

The favorable effect on pregnancy outcome when CBA females are immunized with third party (C57BL) leukocytes seems only to be obtained in the first pregnancy. When immunization is continued into a second pregnancy, the females are found to have the same percentage of fetal resorptions as unimmunized virgin CBA females mated with DBA/2 males. If this effect is due to MHC-encoded antigens in the immunizing cells, it is difficult to understand why the protecting effect on the first pregnancy is associated with a deleterious effect on the second pregnancy.

Two lines of evidence support an interpretation that anti-TLX and not anti-MHC is responsible for the favorable effect in nulliparous and unfavorable effect in multiparous mice. The first is an observation from our laboratory that the fetal resorption rate in unimmunized nulliparous mice falls to a small resorption rate in a second pregnancy, suggesting that the mother is biologically immunized to trophoblast antigens as a consequence of her first pregnancy. The second is that anti-

MHC antibodies are rarely if ever associated with fetal wastage. In contrast, antitrophoblast antibodies have been used to cause abortion in laboratory animals, and cytotoxic anti-TLX antibodies are a common feature of recurrent secondary spontaneous abortion. The CBA \times DBA/2 model has provided interesting information, but its relevance to human pregnancy has yet to be determined.

Research Priorities

The object of this paper has been to discuss several of the currently available and promising new immunobiomarkers for use in reproductive and developmental toxicology. The science of toxicology has been and will be used to assess the impact of certain toxins and/or environmental pollutants on pregnancy outcome. However, as discussed throughout this paper, certain couples are at high risk for reproductive failure. The unwitting inclusion of high risk couples in large epidemiological studies of environmental factors on reproductive performance will cloud the issue of the pathophysiological effects of such environmental factors on normal patterns of reproduction, because such high risk couples are not rare.

It is essential to develop tests that identify, flag, tag, or otherwise signal the at-risk couples in any population about to come under investigation. In this regard, rather simple immunological tests are available, and promising procedures are being developed. It would seem to be a first order research priority to validate or discard existing tests and to direct support toward the development and validation of promising techniques. This would clear a way for research on new techniques which evolve from new methodological and conceptual advances in reproductive immunobiology.

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