THE ROLE OF ALTERED CYTOKINE PATTERN IN THE MAINTENANCE OF PREGNANCY

Ph.D. THESIS

by László Szereday M.D.

Department of Microbiology and Immunology

University Medical School of Pécs

Tutor

Júlia Szekeres-Barthó M.D., Ph.D.

Department of Microbiology and Immunology

University Medical School of Pécs

1998.

TABLE OF CONTENTS

2. Julia Szekeres-Bartho, Par, G., *Szereday, L.,* Smart, C.Y., Achatz, I. Progesterone and non-specific immunological mechanisms in pregnancy. Am. J. Reprod. Immunol. 1997; 38: 176-182.

3. Szekeres-Bartho, J., Faust, Zs., Varga, P., *Szereday, L.*, Kelemen, K. The immunological pregnancy protective effect of progesterone is manifested via controlling cytokine production. Am. J. Reprod. Immunol. 1996; 35: 348-351.

4. *Szereday, L.,* Späth, P., Szekeres-Bartho, J. NK activity and cytokine production of pregnancy lymphocytes after in vitro immunoglobulin treatment. (submitted)

I. LIST OF ABBREVIATIONS

AA	arachidonic acid
BSA	bovine serum albumin
CD	cluster of differentiation
ConA	concanavalin A
CSF	colony-stimulating factor
DAB	diaminobenzidine
ELISA	enzime linked immunosorbent assay
FCS	fetal calf serum
GM-CSF	granulocyte-macrophage colony-stimulating factor
HLA	human leukocyte antigen
HRPO	horse radish peroxidase
hsp	heat shock protein
IFN-γ	interferon γ
IvIg	intravenous immunoglobulin
IL	interleukin
KIR	killer inhibitory receptor
LCT	lymphocyte
MCSF	macrophage colony-stimulating factor
MHC	major histocompatibility antigen
NC	natural cytotoxicity
NK	natural killer
P	progesterone
PAGE	polyacrilamide gel electrophoresis
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PHA	phytohaemagglutinin
PIBF	progesterone-induced blocking factor
PR	progesterone receptor
RSA	reccurent spontaneous abortion
TBC	target binding cell
TBS	tris buffered saline
TCR	T cell receptor
TGF-β	tumor growth factor β
Th	T helper
TNF-α	tumor necrosis factor α

II. INTRODUCTION

1 General overview

Numerous concepts have been proposed to explain the mechanism by which the genetically incompatible fetus may avoid rejection by the maternal immune system. Medawar[1] was the first to formulate the basic problem of human pregnancy: "How does the pregnant mother contrive to nourish within itself, for many weeks or months, a fetus that is an antigenically foreign body ?"

It would seem plausible that non-recognition of fetal antigens by the maternal system favours a normal pregnancy outcome. However, this is not true. In sera of women with successful pregnancies anti-fetal, anti-placental and anti-paternal antibodies are detectable, clearly showing that maternal recognition of fetal antigens does not compromise a pregnancy. Lymphocytes incubated with placentae from normal pregnancies release Th2 cytokines, whereas cells from aborted placentae induce production of Th1 cytokines. Thus, the maternal immune system might not only recognize the pregnancy, but reacts in a differential way resulting in success or failure.

Paternal antigens expressed on fetally derived cells of the placenta might be recognized by the maternal immune system and, indeed, the pregnant female mounts an immune response to these antigens. Chaouat et al.[2] have isolated cytotoxic T cells against paternal MHC-bearing target cells from resorbing embryonic tissue. The

frequent presence of antipaternal alloantibodies in multiparous women also supports this concept.

Several different types of protective mechanisms, e.g., Local immunosuppression in the uterus,[3] suppressor cells,[4] lack of expression of MHC antigens by the trophoblast,[5] immunomodulatory factors[6] and hormones[7,8] have been found to contribute to the success of pregnancy.

The materno-fetal immunological relationship has two aspects:

1. Antigen presentation by the fetus.

2. Maternal recognition of fetally derived antigens and consequent protective immune mechanisms.

2 Presentation of fetally derived antigens. The role of the trophoblast

Essential to pregnancy and to the understanding of immune events at the maternal-fetal interface is the trophoblast. The trophoblast is a tissue of fetal origin and it is in intimate and continuous contact with maternal immunocompetent cells throughout gestation. Thus, the trophoblast should be the interface where fetal antigens are presented to the maternal immune system and also the target of maternal anti-fetal effector mechanisms. Because the trophoblast lines are areas of contact between the fetus and mother, the trophoblast rather than the fetus per se must avoid being targeted by the maternal immune system.

The placenta passively evades immune recognition through the absence of classical class I and II MHC antigens on syncytiotrophoblast cells. However, subsequent studies revealed that invasive cytotrophoblast cells express a nonclassical class I antigen, called HLA-G.[9] This molecule has been suggested to function as a universal "self"-transplantational antigen, preventing maternal immune attack of feto-placental unit. The trophoblast is not sensitive to T cytotoxic lymphocyte mediated lysis, because it does not express classical polymorphic MHC molecules that are indispensable for the recognition of foreign antigens by the cytotoxic T lymphocytes. Neither is the trophoblast sensitive to NK cells.

HLA-G has many unique properties in addition to its tissue distribution, which may effectively modulate maternal tolerance during pregnancy. Although no function has been definitively assigned to HLA-G there is convincing evidence that it provides a

degree of protection for cells from lysis by decidual NK-like cells.[10] Transfection of HLA-G into an HLA-A,B,C, negative cell line rendered partial resistance to lysis by decidual large granular lymphocytes. Interestingly, resistence of the target cells to large granular lymphocytes-mediated lysis was dependent upon expression of trancfected HLA-G reaching a critical level. Nonetheless, expression of HLA-G may enable trophoblast to evade destruction by maternal innate immune system and the degree of expression may be critical to afford this protection.

It is well known that NK cells lyse target cells that do not express MHC products. Recognition of MHC on the target cell activates the killer inhibitory receptor (KIR) of the NK cells and prevents activation. Thus it seems that the monomorphic HLA Class I molecule is used as a kind of mimicry by the trophoblast.

III. MATERNAL IMMUNOREGULATORY MECHANISMS

1. Natural Killer cells

Twenty years ago, it became evident that freshly isolated lymphocytes from normal non-immunized hosts could kill allogenic tumour cell lines.[11] This represented the first evidence for naturally occurring cytotoxic activity against tumour cells in peripherial blood, and was termed natural killer (NK) activity. In contrast to the observed cytotoxic activity caused by T lymphocytes, this natural killing did not need prior sensitization and was MHC-unrestricted.[12] NK cells are important as a first line of defense against infectious agents and metastatic cells.

In normal human pregnancy peripheral NK activity is lower than in nonpregnant individuals, whereas spontaneous abortion is associated with increased systemic NK activity.[13] Resorbing murine embryos are infiltrated with NK cells. Adoptive transfer of high NK activity spleen cells to pregnant mice induces abortion.

NK cells also play a physiological role in the regulation of haematopoesis, where their effects are exerted by cytokine production. Another "homeostatic" role for NK cells may be in reproduction and the control of placentation, as NK cells are abundant at the implantation site in many species.

In humans, uterine NK cells appear to be under hormonal control and they are present during early gestation, particularly at sites where fetal trophoblast cells invade the maternal uterine lining (the decidua). The relationship of uterine NK cells to

circulating NK cells is unclear. If the function of uterine NK cells is to control placentation then they must recognize the semi-allogenic fetal trophoblast cells. Evidence that MHC class I molecules are target ligands for NK cells is now widely accepted. It is therefore relevant that the invading extravillous trophoblast cells in humans express at least two HLA class I molecules: HLA-C and HLA-G. Whether these HLA molecules provide a universal fetal signal to maternal NK cells is still debated. NK cell receptors for class I molecules are of interest to reproductive biologists. Receptors for nonself MHC class I ligands are found, which must be capable of allorecognition.[14]

Obviously, pregnancy is a unique natural scenario where allorecognition occurs physiologically. There has been considerable speculation that NK cells at the implantation site might exert their effect by cytokine production. NK cytokines could influence trophoblast differentation, but also modulate other maternal immune cells such as macrophages or regulate the Th1-Th2 cell bias. There is evidence of a generalized shift of systemic T-cell responses to those of Th2 cells during pregnancy.

2. Cytokines, Th1-Th2 shift

Cytokines play a major role both in the establishment and in the maintaince of normal, human pregnancy. Cytokines may have a beneficial or negative influence on pregnancy outcome depending on the cytokine level present.

In 1986, Mosmann and colleagues reported that most cloned lines of murine CD4+ T cells could be classified into two groups: Th1 and Th2. This was based on the cytokines they produced and their related functional activities.[15] Th1 cells are now defined by their production of interleukin 2 (IL-2), interferon γ (IFN-γ) and tumour necrosis factor α (TNF-α), and Th2 cells are defined by their production of IL-4, IL-5, IL-6, IL-10 and IL-13. Both cell types produce IL-3, TNF-α and granulocyte-macrophage colony-stimulating factor (GM-CSF). Th1 and Th2 clones may have distinct surface markers. For example, all Th2 clones show persistent expression of the CD30 molecule, whereas Th1 clones are consistently CD30 negative.[16]

There is clinical evidence that pregnant women undergo immunological changes consistent with a weakening of cell mediated immunity and strengthening of humoral immunity. Approximately 70% of women with rheumatoid arthritis (a cell-mediated autoimmune disorder) experience a temporary remission of their symptoms during gestation.[17] Systemic lupus erythematosis, in which the principal pathology is mediated by excessive autoantibody production, tends to flare up during pregnancy, especially in women with recently active disease before conception.[18] There are also a number of infectious diseases caused by intracellular pathogens which appear to be

exacerbated by pregnancy. In this category are HIV-associated infections,[19] leprosy,[20] malaria[21] and toxoplasmosis.[22]

Significant IL-10, IL-3 and IL-4 production was detected in the supernatants of placenta and decidua.[23] Lin et al.[24] have demonstrated that Th2-type cytokines, including IL-4, IL-5 and IL-10, were preferentially synthesized at the maternal-fetal interface in all three trimesters of gestation. A shift towards Th2 cytokine production was also noted by Delassus et. al.[25] The primary function of a Th2 response, mediated mainly by IL-10 and IL-4, would be to prevent the stimulation of a strong Th1 response. In fact, it has beeen proposed that an important if not major role for Th2 cells is to subdue or restrain Th1 and phagocyte-dependent immunity.[26] This makes sence considering that Th1-type cytokines can be harmful to pregnancy. Th1-type cytokines clearly have adverse effects on the conceptus in vitro and in vivo. IL-2 and TNF-α are abortifacient in mice.[27,28] There is excessive TNF and IFN-γ in the placenta and decidua of aborting CBA x DBA/2.[29] IFN-γ inhibits trophoblast outgrowth and causes the degeneration of attached blastocysts.[30] IFN-γ and TNF-α inhibit mouse embryonic and fetal development and also the proliferation of human trophoblastic lines in vitro. Yui et al.[31] have shown that TNF-α is cytotoxic to human cytotrophoblast cells which are known to express receptors for TNF-α. The death of trophoblast cells is caused by apoptosis and they suggest that TNF-α, augmented by IFN-γ, may bring about a premature depletion of progenitor trophoblast cells which may finally result in intrauterine damage. In vivo inflammatory cytokines such as IL-2, TNF-α, and IFN-γ can terminate normal pregnancy when injected into pregnant

mice.[32] These data nevertheless led to the concept that "successful allopregnancy is a Th2 phenomenon".[33]

3. Progesterone-dependent immunomodulation

Progesterone (P) is essential for the maintaince of pregnancy in a number of mammalian species. High concentrations of progesterone prolong the survival of xenogenic and allogenic grafts[34,35] and this hormone affects various phases of the immune response in vitro[36,37] Many publications reported that P blocks T cell activation in concentrations of 5 to 20 μg/ml.[38] Stites et al.[37] reported on different mechanisms resulting in T cell activation blocking by P and cortisol. The inhibitory effect of cortisol seems to involve monocytes, while P has a direct effect on T cells. T lymphocyte proliferation requires the presence of a growth factor, interleukin-2. In most investigations dealing with in vitro effects of P on lymphocytes reactivity, only supraphysiological (0.5-20 μg/ml) doses were found to be effective. Thus, it was concluded that P might have a role as a natural immunosuppressant during pregnancy, although its action was assumed to be restricted to the materno-fetal interface where P concentrations reach the high level required for in vitro blocking.[39] This proposed mechanism would be favourable because it suggests a reduced maternal response to fetal antigens in the placenta. On the other hand, lower concentrations would not jeopardize immunity of the mother. Progesterone at physiological concentrations inhibited natural cytotoxic (NC) activity in a dose-

related manner and an inverse relationship was found between P concentration and cytotoxic activity of the lymphocytes.[40] Preincubation of the lymphocytes with P depleted pregnancy serum did not result in significant inhibition on cytotoxic activity. In fact, absorption with anti-progesterone antibody caused an 80% decrease on cytotoxic activity of pregnancy sera. Lymphocytes of healthy pregnant women are more sensitive to the NC blocking of P because they have significantly higher P binding capacities than those from nonpregnant individuals or pregnant women at risk for premature pregnancy termination.[41] Earlier investigations from our laboratory demonstrated progesterone-receptors (PR) in normal pregnancy lymphocytes but not in nonpregnant lymphocytes.[42] Lymphocyte PRs do not seem to be identical with the classical P binding sites. PRs appear in peripherial blood lymphocytes as early as the tenth day of gestation and disappear during term and preterm labour as well as during spontaneous abortion.[43]

Biological effects of progesterone are manifested via a 34 kDa protein named the Progesterone Induced Blocking Factor (PIBF), which is released by lymphocytes of healthy pregnant women in the presence of progesterone. [44]

PIBF has pleiotropic immunomodulatory properties: it blocks NK mediated cell lysis as well as natural cytotoxicity, strongly depresses the mixed lymphocyte reaction, and by blocking NK activity and modifying cytokine production it exerts anti-abortive effect in mice.[45,46]

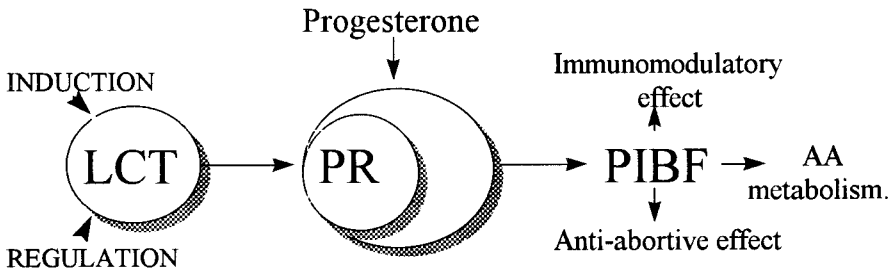
PIBF can be detected in sera of pregnant women and its concentration is higher in the sera of healthy pregnant women than in those of non-pregnant individuals or

pregnant women with symptoms of threatened abortion. Similar results were found by testing PIBF expression on lymphocytes of healthy pregnant women and those of women with pathological pregnancies.[47]

PIBF has been shown to inhibit Th1 type responses, e.g. alloantigen and mitogen-induced proliferation, as well as NK activity. More recent data provided evidence that PIBF alters the profile of cytokine secretion by activated lymphocytes. In supernatants of murine spleen cells activated in the presence of PIBF the concentration of IFNγ was not substantially different from controls. However, the same spleen cells produced significantly more IL-10, IL-3 and IL-4 than those cultured without the progesterone-dependent protein.[48] These data indicate that PIBF alters the Th1/Th2 balance and via altered cytokine ratios it contributes to decreased cell-mediated responses during pregnancy.

Among other effects PIBF displays an antiabortive effect in mice.[45] In mice there is direct evidence for the role of high NK activity in pregnancy termination. Resorption sites in mice are infiltrated by NK cells.[49] Modulation of natural killer activity influences resorption rates.[50]Adoptive transfer of high NK activity spleen cells into pregnant mice induces abortion.[51] Simultaneous PIBF treatment of pregnant mice corrects the abortive effect of NK activity.[52] PIBF treatment also prevented progesterone receptor block induced abortion. Neutralisation of endogenous PIBF with a specific antibody resulted in pregnancy termination in mice suggesting that, at least in mice, PIBF is indispensable for a successful gestation.

The schematic representation of the progesterone-dependent immunomodulatory pathway is described in Figure 1.



PROGESTERONE-DEPENDENT IMMUNOMODULATION
DURING PREGNANCY

Fig. 1.

Recognition of fetally derived antigens results in induction of P binding sites in pregnancy lymphocytes. Receptor binding of P in the lymphocytes will induce the release of a protein called the progesterone-induced blocking factor (PIBF). This protein will initiate a series of immunological events leading to an impairment of NK and cytotoxic activity. Finally, inhibition of NK activity will result in the prolongation of pregnancy.

4. The role of γδ T lymphocytes during pregnancy

Most peripheral T cells express a TCR which consists of a heterodimer made up of an α and a β polypeptide chain. About 5% of peripheral T lymphocytes in humans and in mice[53] express another type of TCR heterodimer made up of a γ and a δ polypeptide chain. Similarly to αβ TCR+ cells, γδ TCR+ cells depend on the thymus for maturation[54] and express T cell markers such as CD2, CD3, CD5,CD45 and CD11b.[55] The response of γδ cells to their ligands is similar to that of αβ cells. It can be the killing of the target cells and the production of lymphokines such as IL-2. A major population of γδ TCR lymphocytes resides in the skin, intestine, lung, and uterus of mice.

γδ T lymphocytes at the maternal-fetal interface are maternally derived. Relative to the total T lymphocyte population, the percentage of γδ TCR-bearing T lymphocytes at the maternal-fetal interface are enriched three- to fourfold compared with maternal spleen, and twofold compared with nonpregnant uteri.[56] In terms of absolute numbers the estimated γδ T cells are increased nearly 100-fold in pregnant animals compared with nonpregnant animals.

Human decidua of early pregnancy has rich vascularization and is mainly composed of three cell types: 1. the cuboidal cells of the glandular epithelium lining the endometrial glands; 2. the large stromal cells; and 3. leukocytes. The γδ T cells were localized in cell aggregates, intraepithelially, as well as in the stroma.[57] Decidua forms the foremost maternal border to the fetus and is penetrated by the extravillous

throphoblasts moving towards blood vessels during the formation of placenta. Obviously, the lymphoid tissue in the decidua is there to meet certain specific requirements. On one hand, a genetically different implanted fetus must be immunologically accepted and the development of the placental bed must be allowed. On the other hand, the invasiviness of the trophoblast while forming the placenta must be controlled. Four different lymphocyte populations of similar size were identified in decidua: 1. TCRγδ+/CD56+ cells 2. TCRγδ+/CD56- cells 3. TCRγδ-/CD56+ cells 4. TCRαβ+/CD8+ cells. The functions of either one of these populations might be both regulative, i.e., down-regulating other cell populations to prevent immunologic reactions against the fetus, and protective, i.e., protecting the uterus from extensive and unwanted invasion of the extravillous trophoblast when forming the placenta.

Murine γδ T lymphocytes are relatively abundant in the intraepithelial lymphocytes of the uterus[58]. There have been several lines of evidence that γδ T cells in an activated state are present in an increased number at the maternal-fetal interface during pregnancy in human,[57] sheep and mouse, suggesting that γδ T cells in uterine intraepithelial lymphocytes may contribute to maternal antifetal immune responses at the maternal-fetal interface.

There have been several lines of evidence that γδ T cells recognize allogenic MHC antigens including class I, class II, and class Ib such as Qa and TL. The trophoblast does not express classical MHC class I and class II gene products but does express minimally polymorphic nonclassical molecules known as HLA-G in humans. Recently, Schild et al.[59] have shown that γδ T cells specific for MHC class II IE^k or TL

can recognize the antigens directly without the need for specialized antigen-presenting cells. More recently, Heyborne et al.[60] have clearly demonstrated that mouse γδ T cells bearing Vγ1 chain, which are specialized to recognize heat-shock protein 60 (hsp60), can recognize trophoblasts from β2-microglobulin-deficient mice and even from the human trophoblast cell line. Therefore, it is alternatively possible that the γδ T cells in uterine intraepithelial lymphocytes may recognize phylogenically conserved mammalian molecules such as heat shock protein on trophoblasts and can be triggered for activation during pregnancy.

As specific constituents of the reproductive tract, γδ T lymphocytes may be involved in regulating a variety of physiologic and pathophysiologic events in reproductive biology. For instance, inadequate invasion by trophoblast into the maternal spiral arteries characterizes preeclampsia and intrauterine growth retardation.[61] Specific recognition of trophoblast antigen by γδ T cells might affect this process.

5. Human pregnancy pathology

The inability to maintain a pregnancy is a considerable health problem for many couples trying to build a family.

It is well established that many pregnant women do not reject their embryos; what remains controversial, however, is to what extent an aberrant immune response to the pregnancy is responsible for those instances in which the fetus is lost. Spontaneous abortion occurs in approximately 15% of all clinically apparent pregnancies.[62] Reccurent spontaneous abortion (RSA) may affect 1% of couples.[63] RSA is defined as two or more consecutive pregnancy losses under 20 weeks of gestation. Two recent British series of RSA patients agree that ~80% of such couples do not have recognizable abnormalities (chromosomal translocation, autoimmunity, etc.) which could account for pregnancy loss. Reccurently aborting couples share significantly more HLA antigens than control couples,[64] although no particular specificity or haplotype is involved. There are data which suggest that immunization using paternal leukocytes or third-party leukocytes significantly increases the success rate.[65] Moreover, one of the few prognostic factors that has consistently been confirmed is the increasing risk of future miscarriage in relation to the number of previous miscarriages. This influence is weak: the probability of a successful next pregnancy decreases by only 15% for each previous loss.[65]

IV.AIMS OF THE STUDY AND RESULTS

It is well established that pregnancy is characterized by an altered cytokine balance. Previous data from this laboratory suggest an effect of PIBF on the cytokine production pattern.

The aim of this study was to investigate the role of cytokines in pregnancy termination and to elucidate the relationship between progesterone-dependent immunomodulation and cytokine production. This relationship was investiagted in both in vitro and in vivo systems.

1. In vitro studies

1) Cytokines are of particular importance in communication between the fetus and maternal cells. Pregnancy is associated with a relative increase in Th2-associated immunity, characterized by increased production of the cytokines IL-4 and IL-10.[23] These changes occur concomitantly with increased immunoglobulin production and decreased Th1 action.

Our earlier data suggest that immunological effects of PIBF are manifested via cytokine mediated pathways and induce a Th2 shift.[48]

In vitro studies revealed that PIBF induces a Th2 type cytokine production by activated lymphocytes. Activated murine spleen cells cultured in the presence of PIBF

released significantly more IL-10 as well as IL-3 and IL-4 than those without PIBF. (Paper 2.)

2) Increased NK activity of pregnancy lymphocytes after neutralization of endogenous PIBF activity is corrected by anti IL-12 treatment. PIBF inhibits IL-12 production by activated lymphocytes (Paper 3.)

Our earlier studies revealed that NK activity is low in pregnancy and spontaneous pregnancy termination is accompanied by an elevated NK activity.[66] PIBF strongly inhibits NK activity.[67]

In order to investigate the role of altered cytokine pattern in the NK inhibitory effect of PIBF, we treated lymphocytes of pregnant women with anti-PIBF neutralizing antibody and determined NK activity.

Neutralization of endogenous PIBF activity by a specific antibody in vitro, as well as anti-IL-10 treatment of lymphocytes, results in increased NK activity and this is corrected by simultaneous administration of IL-10. Addition of exogenous IL-10 corrected the effect of PIBF on NK activity. PIBF was also able to overcome the NK stimulating effect of anti-IL-10 treatment, suggesting that PIBF uses other than the IL-10 pathway (IL-10 synthesis and release) to inhibit NK activity. This concept is supported by the finding that anti-IL-12 treatment inhibited increased NK-mediated lysis due to anti-PIBF treatment. This is in line with the observation that in the presence and not in the absence of PIBF, do mitogen activated lymphocytes of non-pregnant individuals produce less IL-12. (Paper 3.)

2. In vivo studies

Nonspecific immunological mechanisms play a key role in pregnancy loss both in mice and humans.[68] In normal human pregnancy, NK activity is significantly lower than in nonpregnant individuals and there is a significant increase of NK activity before spontaneous pregnancy termination.[69] There is direct evidence for the role of high NK activity in pregnancy termination in mice. Resorption sites in mice are infiltrated by NK cells.[70] Modulation of NK activity influenced resorption rates[71] and murine abortions can be prevented by an anti-NK antibody. Szekeres-Bartho at al.[72] have shown that PIBF blocks NK activity *in vitro* and prevents resorptions induced by transfer of spleen cells with high NK activity.[45]

In order to explain the role of an altered cytokine pattern in the NK inhibitory and anti-abortive effect of PIBF, we treated pregnant mice with anti-PIBF neutralizing antibody and investigated the *in vivo* effect of PIBF on cytokine production as well as the relationship between cytokine production, NK activity, and pregnancy loss. (Paper 3.)

Neutralization of endogenous PIBF in pregnant mice resulted in an increased percentage of IFN-γ-positive spleen cells. There was a positive relationship between the percentage of IFN-γ-positive spleen cells and NK activity. In animals with a high resorption rate, the splenic IFN-γ expression rate was significantly higher than in those with a low resorption rate. (Paper 3.)

In mice treated with anti-PIBF, splenic IL-10 production was significantly lower than in mice treated with the same amount of normal rabbit serum or untreated mice of similar gestational age. Splenic IL-10 production, on the other hand, was inversely related to resorption rates.

IL-10 inhibits cytokine production by Th1-type cells as well as CD8+ T cells.[73,74] Therefore, the lack of IL-10 production might be one of the factors responsible for high NK activity.

We treated another group of mice with anti-PIBF neutralizing antibody simultaneously with anti-NK or anti-NC monoclonal antibodies. (Paper 2.)

This treatment resulted in a significant decrease in the percentage of IFN-γ-positive spleen cells.

Anti-NK treatment corrected the decreased splenic IL-10 production in anti-PIBF treated mice.

In the present experiments neutralization of NK and NC activity in anti-PIBF treated mice corrected not only resorption rates but also cytokine values. That implies that NK cells are not simply targets of cytokines, thus the abortive effect of anti-PIBF treatment is not merely due to lymphokine-activated killer cells, but these cells are producers of cytokines themselves. In mice IL-10 is produced by Th2 cells as well as by macrophages and B cells. IFN-γ is produced by NK cells. (Paper 2.)

3. Human studies

Previously we showed that PIBF facilitates the production of Th2 type cytokines in activated lymphocytes. This study investigated the *in vivo* relationship between progesterone-dependent immunomodulation and cytokine production of pregnancy lymphocytes. We found low IL-12 with increased PIBF and IL-10 expression on lymphocytes from healthy pregnant women. The cytokine production pattern was related to the presence or absence of previous abortions as well as to the outcome of pregnancy.

Sera of women at risk for premature pregnancy termination contained significantly higher concentrations of TNF-α than those from healthy pregnant women and PIBF expression on the lymphocytes was inversely related to serum concentration of TNF-α. (Paper 3.) The aim of our study was to test the hypothesis that lymphocytes of women at risk for premature pregnancy termination (habitual aborters as well as women showing clinical symptoms of threatened abortion or threatened preterm delivery) produce Th1-type cytokines following the loss of PIBF production and that lymphocytes of healthy pregnant women produce Th2-type cytokines when PIBF is present in the lymphocytes. (Paper 1.)

Our data revealed an increased expression of IL-12 on lymphocytes from women with a history of recurrent abortions or with clinical symptoms (bleeding or regular uterine contractions) of threatened abortion or threatened preterm delivery,

whereas lymphocytes from normal healthy pregnant women expressed IL-10 together with PIBF instead.

The present data provide further evidence that PIBF may induce a Th2-type cytokine response beneficial for pregnancy, whereas many women with pathologic pregnancies and low PIBF production manifest an abnormally high Th1 response during pregnancy.

We also investigated the relationship between *in vivo* cytokine production of pregnancy lymphocytes and the outcome of pregnancy. (Paper 1.)

The findings of this study revealed a relationship between clinical symptoms of premature pregnancy termination (uterine contractions and bleeding) and the cytokine production of pregnancy lymphocytes. Lymphocytes of pregnant women showing clinical symptoms of threatened premature pregnancy termination expressed significantly more IL-12, whereas IL-10 expression was significantly lower than in asymptomatic patients. These data imply that cytokine determination might be a potential diagnostic tool, provided it predicts premature pregnancy termination with a higher precision than clinical symptoms.

Cytokine expression in the lymphocytes from women with a history of three or more unexplained spontaneous abortions and no successful pregnancy were tested for IL-10 and IL-12 production. We found an increased IL-12 and a decreased IL-10 expression on peripheral lymphocytes from women with a history of recurrent abortions compared to healthy pregnant women. (Paper 1.)

Retrospective comparison of cytokine expression in lymphocytes of women whose pregnancies resulted in term labour with those of women whose pregnancies terminated prematurely revealed a significantly higher IL-10 expression and a slightly lower IL-12 expression in successful pregnancies. (Paper 1.)

Our data together with earlier findings indicating that PIBF promotes the production of Th2 type cytokines in vitro suggest that during normal pregnancy PIBF might affect cytokine production.

Tumor necrosis factor is amongst those cytokines considered critical in pregnancy. On the one hand it appears that TNF at physiological concentrations favor successful reproduction. On the other hand, several lines of evidence suggest that tha same cytokine at pathologically increased levels may compromise pregnancy. Excess TNF may cause placental injury[75] and abortion,[76] and elevated levels of TNF[77] have been implicated in the initiation of preterm labor. We measured the quantity of TNF-α in human serum of healthy pregnant women and in women at risk for premature pregnancy termination by measuring cytotoxicity to L929 cells. Furthermore we determined the expression of PIBF on lymphocytes of the same pregnant women by immunocytochemistry.(Paper 3.)

Our results showed that the concentration of TNF-α was significantly higher in sera of pregnant women at risk for premature pregnancy termination than in those of healthy pregnant women. At the same time PIBF expression on the lymphocytes was inversely related to serum concentrations of TNF-α. (Paper 3.)

Treatment for recurrent miscarriage has usually been given to all women with three or more abortions of unknown cause. As these patients have a 50-60 % subsequent live birth rate, no treatment has been shown to unequivocally improve the live birth rate. Immunoglobulin is the latest treatment to be applied. The preliminary studies on intravenous immunoglobulin (IvIg) to prevent recurrent miscarriage have been encouraging.[78,79] IvIg is prepared from pooled plasma from thousands of healthy donors.

In a preliminary trial Carp et al.[80] used intravenous immunoglobulin treatment in patients considered to have such a poor prognosis that they have little chance of even sustaining a pregnancy to viability. Twelve patients were treated, ten conceived. Five have had subsequent live birth. This is still too small of a group from which to draw definite conclusions about the efficiency of immunoglobulin to prevent abortion. However, five births in ten patients is an encouraging result, especially when the expected poor obstetric outcome is considered.

Intravenous immunoglobulin has been reported to be three times as efficient as paternal leukocyte immunization,[81] but others claimed it to be ineffective and have abandoned its use.[82]

Whatever the precise pathophysiologic mechanism of immunologically mediated RSA is, there is a good chance that the infusion of intravenous immunoglobulin will prove beneficial. Among the many immunomodulatory effects proposed for IvIg are the neutralization of circulating autoantibodies, the blockade of Fc receptors, the inhibition of complement-mediated cytotoxicity, the modulation of

release of cytokines from lymphocytes, and the selection of T cell repertoires.[83,84] Many of these actions will tend to decrease NK cytotoxicity, divert T-cell responses from Th1 pattern toward a Th2 pattern, and decrease autoantibody titers: all mechanisms of potential benefit in RSA. However, the rationale of this treatment is not known.

In order to elucidate the therapeutic mechanism of intravenous immunoglobulin treatment, we treated pregnancy lymphocytes of women with recurrent miscarriage by immunoglobulin and investigated the IL-10 and IL-12 production of pregnancy lymphocytes using immunocytochemistry. We hypothesized that via increasing IL-10 and inhibiting IL-12 production, immunoglobulin corrects the altered cytokine pattern to prevent abortion. (Paper 4.)

We found a significantly increased expression of IL-10 and a significantly decreased expression of IL-12 in immunoglobulin-treated peripheral lymphocytes from women with a history of recurrent abortions or with clinical symptoms of threatened abortion or threatened preterm delivery, whereas lymphocytes from normal healthy pregnant women showed only slightly altered cytokine production.

A significantly increased NK activity has been associated with RSA. It is possible that elevated NK activity is due to increased IL-12 production.

We demonstrated a decreased IL-12 production together with a decreased NK activity in RSA lymphocytes after in vitro immunoglobulin treatment. (Paper 4.)

Our finding that immunoglobulin treatment reduces NK activity and thus favors a successful pregnancy is consistent with the observation that cytotoxic activity

induced by Th1 responses have deleterious effects on pregnancy and that the prevention of such responses protects pregnancy. Therefore, women at risk for premature pregnancy termination and elevated NK activity may benefit from immunoglobulin treatment because immunoglobulin not only reduces the NK activity as previously reported,[85] but also inhibits the Th1 responses and helps to shift the immune response towards a protective Th2 response. (Paper 4.)

V. PAPERS

PAPER 1.

AJRI 1997; 38:418–422
Printed in the United States of America - all rights reserved.

American Journal of Reproductive Immunology
ISSN 8755-8920

Cytokine Production by Lymphocytes in Pregnancy

L. SZEREDAY, P. VARGA, AND J. SZEKERES-BARTHO

Szereday L, Varga P, Szekeres-Bartho J. Cytokine production by lymphocytes in pregnancy. AJRI 1997; 38:418–422 © Munksgaard, Copenhagen

PROBLEM: In the presence of progesterone lymphocytes of pregnant women release a 34-kDa protein named the progesterone-induced blocking factor (PIBF). PIBF mediates the immunomodulatory and anti-abortive effects of progesterone and its presence is related to the outcome of pregnancy. PIBF induces production of Th2 type cytokines by activated lymphocytes. The in vivo relationship between PIBF- and cytokine production of pregnancy lymphocytes and the outcome of pregnancy was investigated.

METHOD OF STUDY: Interleukin (IL)-12 and IL-10 production and PIBF expression in peripheral lymphocytes of 111 healthy pregnant women and 120 women at risk for premature pregnancy termination were detected by immunocytochemistry.

RESULTS: We found increased IL-12 and low PIBF and IL-10 expression on lymphocytes of "risk" patients, and a high rate of IL-10 and PIBF positivity on lymphocytes from healthy pregnant women. The cytokine production pattern of the lymphocytes was related to the presence or absence of previous abortions as well as to the outcome of pregnancy.

CONCLUSION: These data suggest the involvement of an altered cytokine production pattern in the immunologic effects of progesterone.

Key words:
IL-10, IL-12, progesterone

L. SZEREDAY
P. VARGA
J. SZEKERES-BARTHO
Department of Microbiology,
University Medical School of Pecs,
Pecs, Hungary

Address reprint request to
Julia Szekeres-Bartho, M.D., PhD,
Department of Microbiology,
University Medical School of Pecs,
H-7643 Pecs, Hungary.

Submitted April 30, 1997; accepted
June 20, 1997.

INTRODUCTION

Progesterone is essential for normal gestation in humans.[1,2] Earlier observations called attention to the importance of progesterone in immunomodulation during pregnancy.[3] Lymphocytes of healthy pregnant woman exhibit a higher sensitivity to progesterone and a higher progesterone binding capacity than normal non-pregnancy lymphocytes.[4] These differences are caused by the presence of progesterone receptors in pregnancy lymphocytes, but not in non-pregnancy lymphocytes.[5] In the presence of progesterone, receptor-positive lymphocytes release a 34-kDa protein, named the progesterone-induced blocking factor (PIBF).[6] PIBF is endowed with immunomodulatory [7] and anti-abortive properties[12] and it inhibits the release of arachidonic acid[6]; the capacity of the lymphocytes to produce PIBF is related to the outcome of pregnancy.[8]

PIBF exerts a strong anti-natural killer (NK) activity.[7] Increased NK activity is related to pregnancy termination. Murine resorptions are infiltrated with cells of the NK phenotype,[9] and modulation of NK activity affects the outcome of pregnancy in mice.[10,11] PIBF prevents resorptions induced by transfer of high NK activity spleen cells.[12] In normal human pregnancy, NK activity is significantly lower than in non-pregnant individuals, whereas idiopathic spontaneous abortions and term labor are associated with

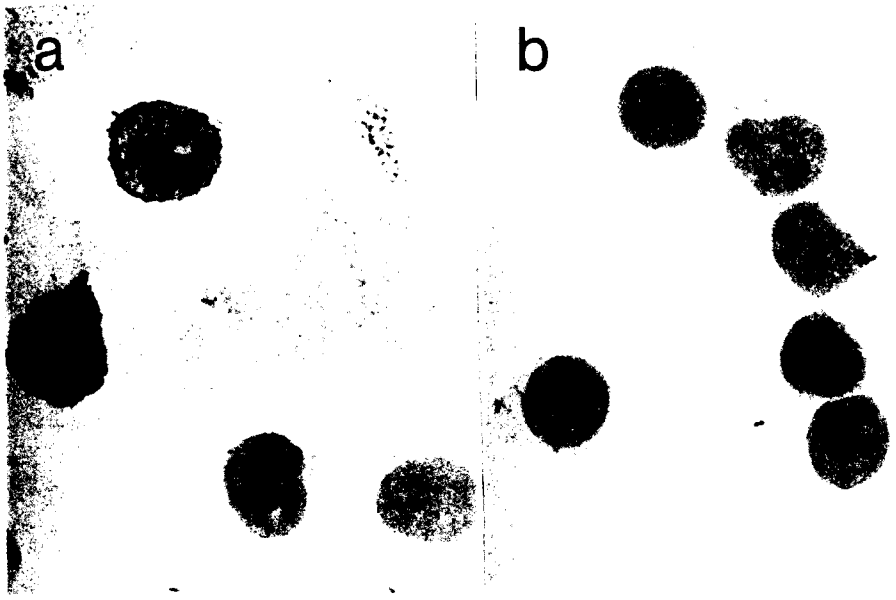


Fig. 1. IL-12 expression on lymphocytes of pregnant women. (**a**) IL-12 positive lymphocytes; (**b**) IL-12 negative lymphocytes (silver intensification, magnification ×1000).

increased NK activity.[13] NK activity is regulated by cytokines. IL-2, IL-7, IL-12, and IL-6 have been shown to exert a direct and potent stimulatory effects on NK activity.[14]

Cytokines are of particular importance in mediating communications between the conceptus and maternal cells. Cell-mediated immunity is decreased during pregnancy,[15] whereas T-cell–dependent immunoglobulin production remains intact or increases.[16,17]

Many observations support the concept that pregnancy is associated with an altered Th_1/Th_2 balance. Pregnancy is associated with a relative increase in Th2-associated immunity, characterized by increased production of the cytokines IL-4 and IL-10.[18] These changes occur concomitantly with increased immunoglobulin production and decreased Th1 action.

Our earlier data suggest that immunologic effects of PIBF are manifested via cytokine-mediated pathways and induce a Th2 shift.[19] In the presence of PIBF, activated lymphocytes produce significantly more IL-10, IL-3, and IL-4, than those cultured without the progesterone-induced protein.

We tested the hypothesis that lymphocytes of women at risk for premature pregnancy termination (habitual aborters as well as women showing clinical symptoms of threatened abortion or threatened preterm delivery) produce Th1-type cytokines after the loss of PIBF production and that lymphocytes of healthy pregnant women produce Th2-type cytokines when PIBF is present in the lymphocytes. We also investigated the relationship between in vivo PIBF and cytokine production of pregnancy lymphocytes and the outcome of pregnancy.

MATERIALS AND METHODS

Patients

Two hundred and thirty-one pregnant women were included in this study. The control group included 111 healthy pregnant women. The "patient" group consisted of 120 women at risk for premature pregnancy termination (Table 1). The latter group included primary habitual aborters (n = 47) as well as women showing clinical symptoms (bleeding or regular uterine contractions) of threatened premature pregnancy termination. Primary habitual aborters were defined as those with a history of three or more unexplained spontaneous abortions and no successful pregnancy.

Immunocytochemistry

Lymphocytes were isolated from heparinized venous blood on Ficoll-Paque gradient. The cells were washed once in Parker 199 solution and centrifuged on glass microscope slides. The slides were dried at room temperature, the cells were fixed for 5 min in cold acetone and washed in Tris-buffered saline (TBS). All incubations were carried out at room temperature in a humid chamber. After blocking of endogenous peroxidase activity with 1% H_2O_2 the cells were further incubated in TBS containing 1% bovine serum albumin (BSA, Sigma Chemical Co., St. Louis, MO) for blocking nonspecific protein binding sites.

TABLE I. Cytokine Expression on Lymphocytes of Pregnant Women of Different Gestational Ages

	Weeks of gestation		
	>20	<20	
Normal pregnancy			
No. of patients (n = 111)	93	18	
IL-10	28.1 ± 2.17	33.1 ± 6	NS
IL-12	6.63 ± 1.13	2.9 ± 1	NS
Pathologic pregnancy			
No. of patients (n = 120)	51	69	
IL-10	7.3 ± 1.3	6.3 ± 1.2	NS
IL-12	31.8 ± 4.8	28.3 ± 3.8	NS

The primary antibodies were polyclonal anti-PIBF IgG (prepared in this laboratory [20]) and anti-IL-10 and anti-IL-12 polyclonal antibodies (purchased from (R&D Systems, Abingdon, Oxon, UK). The antibodies were diluted 1:100, 1:50, and 1:50, respectively, in TBS supplemented with 0.5% BSA. The second antibodies (HRPO-labelled anti-rabbit, anti-goat and anti-goat IgG) were purchased from Dakopatts and Sigma, Hungary, and applied at dilutions of 1:200, 1:100, and 1:100, respectively, for 30 min. The reaction was developed by diaminobenzidine and intensified with silver staining.

The percentage of positive cells was calculated after counting 300 lymphocytes in the microscope at high-power magnification.

Statistics

The two-tailed Student's *t*-test was used for statistical evaluation of the data. Differences were considered significant if the *P* value was equal to or less than 0.05.

RESULTS

To investigate the relationship between PIBF and cytokine production and the outcome of pregnancy, we determined the rate of PIBF, IL-10, and IL-12 positive cells among peripheral lymphocytes of healthy pregnant women and women at risk for premature pregnancy termination.

PIBF, IL-10, and IL-12 Expression on Lymphocytes of Healthy Pregnant Women and of Those at Risk for Premature Pregnancy Termination

Because we found no significant difference between lymphocyte cytokine expression before or after the 20th week of gestation within the groups (Table I), we considered it adequate to compare the group of normal pregnancies with that of pathologic pregnancies.

In the peripheral blood of healthy pregnant women the percentage of PIBF positive lymphocytes was 65.05 ± 3.15%. In 120 women with pathologic pregnancies (habitual aborters as well as women showing clinical symptoms [bleeding or regular uterine contractions] of threatened premature pregnancy termination) we found a significantly lower ($P < 0.001$) rate of positivity (14.52 ± 2.5%).

The percentage of IL-10+ lymphocytes in the peripheral blood of healthy pregnant women was 28.54 ± 2.06%. In women with pathologic pregnancies we found a significantly lower ($P < 0.001$) rate of positivity (7.06 ± 0.94%) (Fig. 2a).

The percentage of IL-12+ lymphocytes in peripheral blood of healthy pregnant women was 6.34 ± 1.05%. In women with pathologic pregnancies we found a significantly higher ($P < 0.001$) rate of positivity (30.59 ± 2.92 %) (Fig. 2a).

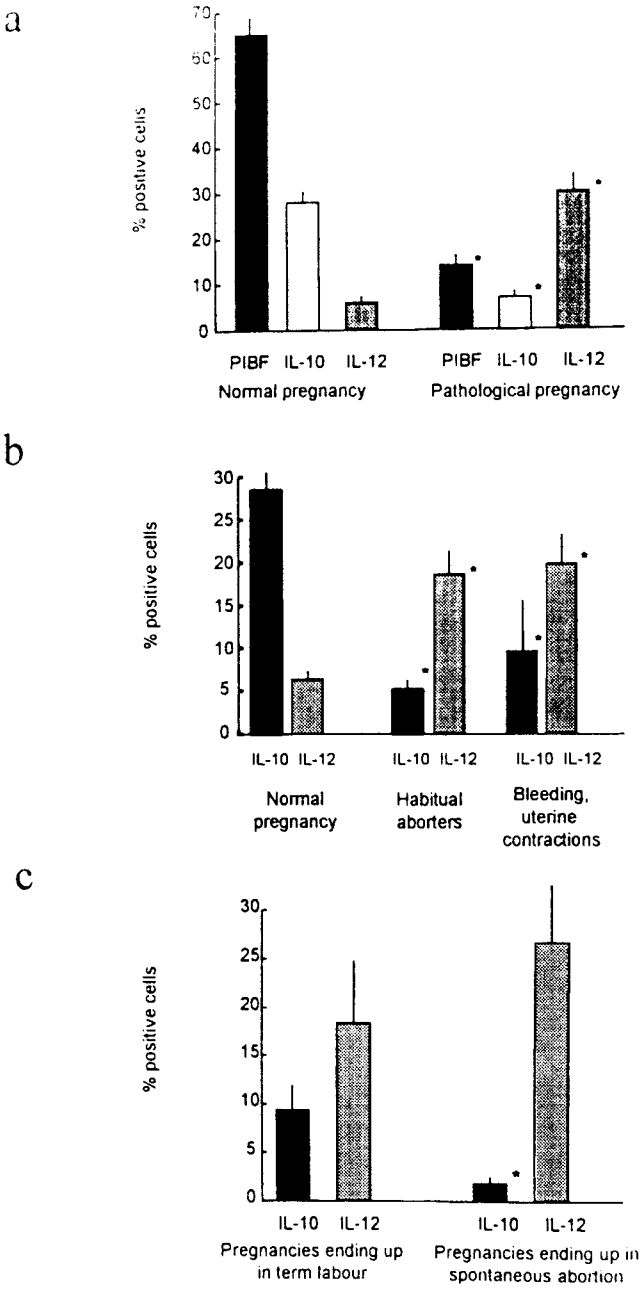


Fig. 2. (**a**) PIBF, IL-10, and IL-12 expression on lymphocytes of women with normal and pathologic pregnancies. The bars represent the mean ± SEM of 111 (normal pregnancy) and 120 (pathologic pregnancy) determinations. * $P < 0.001$. (**b**) The relationship between clinical history and cytokine production. The bars represent the mean ± SEM of 111 (normal pregnancy), 30 (women with a history of three or more unexplained spontaneous abortions and no successful pregnancy), and 14 (women with clinical symptoms of bleeding and uterine contractions) determinations. * $P < 0.001$. (**c**) The relationship between cytokine pattern and the outcome of pregnancy. The bars represent the mean ± SEM of 7 (pregnancies ending in term labor) and 8(pregnancies ending in spontaneous abortion) determinations. *$P < 0.001$.

Relationship Between Clinical Symptoms and Cytokine Expression

Lymphocytes of 14 women with clinical symptoms of premature pregnancy termination (bleeding or uterine contractions) were tested for IL-10 and IL-12 production. The percentage of IL-10 positive cells in the peripheral blood of women with clinical symptoms at the time of sampling was significantly ($P < 0.001$) lower (9.4 ± 6.4), whereas IL-12 production was significantly ($P < 0.001$) higher (19.4 ± 3.0), than the percentage in healthy pregnant woman (Fig.2b).

Relationship Between Clinical History and Cytokine Production

Lymphocytes of 30 pregnant women with a history of three or more unexplained spontaneous abortions and no successful pregnancy were tested for IL-10 and IL-12 production. The percentage of IL-10 positive cells in the peripheral blood of women with a history of recurrent abortions was significantly lower (5.13 ± 1.52), whereas the percentage of IL-12 positive cells was significantly higher (18.53 ± 3.73) than in healthy pregnant woman. (Fig.2b).

Relationship Between the Cytokine Pattern and the Outcome of Pregnancy

Cytokine expression in the lymphocytes from women whose pregnancies terminated prematurely were compared with those of women with a normal outcome. During gestation we found a significantly ($P < 0.001$) higher rate of IL-10 positive cells (9.4 ± 2.03) in women with successful pregnancies, than in those whose pregnancies ended in miscarriage (1.75 ± 0.625). In pregnancies resulting in spontaneous abortion we found a higher rate of IL-12 positive cells during pregnancy than in those ending in term labor, but these differences were not statistically significant (Fig.2c).

DISCUSSION

Pregnancy is characterized by a Th2 bias. Th1 cytokines (IL-2, IL-12, and IFNγ) have been shown to induce pregnancy termination.[21] The protection of the fetus is based on increased secretion of Th2 cytokines (IL-4, IL-5, and IL-10) which down-regulate potentially deleterious strong cell-mediated responses.

Maternal immune recognition of antigens derived from the fetus results in the release of cytokines that promote the growth of the placenta.[22] The extent of stimulation of maternal strain type lymphocytes in response to stimulator placental cells in mixed lymphocyte-placenta reactions (MLPR) was much higher in the normal mating combination than in abortion-prone mating combination.

Chaouat et al.[23] have also shown that alloimmunization enhances the placental production of IL-4 and IL-10 in the resorption-prone CBA/J×DBA/2 matings.

Lin et al.[18] detected Th2-specific cytokines IL-3, IL-4, IL-5, and IL-10 in cell supernatants derived from fetal-placental units from normal murine pregnancies in all three trimesters of gestation.

Previously we showed that PIBF facilitates the production of Th2 type cytokines in activated lymphocytes.[19] This study investigated the in vivo relationship between progesterone-dependent immunomodulation and cytokine production of pregnancy lymphocytes. We found an increased expression of IL-12 on peripheral lymphocytes from women with a history of recurrent abortions or with clinical symptoms (bleeding or regular uterine contractions) of threatened abortion or threatened preterm delivery, whereas lymphocytes from normal healthy pregnant woman expressed IL-10 instead, together with PIBF.

NK activity plays a role in spontaneous termination of pregnancy in mice. Resorption sites in mice are infiltrated by NK cells.[9] Modulation of NK activity influenced resorption rates in mice.[10] PIBF blocks NK activity in vitro[6] and prevents resorptions induced by transfer of spleen cells with high NK activity[12]; furthermore, pregnancy loss induced by anti-PIBF treatment could be prevented by anti-NK neutralizing antibody. However, the mechanisms by which these effects are achieved are not clear. Because IL-12 has been shown to stimulate NK activity (which could damage the trophoblast and compromise fetal survival), it could play a role in reproductive dysfunction in patients with pathologic pregnancies.

The present data provide further evidence that PIBF may induce a Th2-type cytokine response beneficial for pregnancy, whereas many women with pathologic pregnancies and low PIBF production manifest an abnormally high Th1 response during pregnancy.

Our data revealed a relationship between clinical symptoms of premature pregnancy termination (bleeding or uterine contractions) and the cytokine production pattern. Lymphocytes of pregnant women with threatening clinical symptoms expressed significantly more IL-12, whereas IL-10 expression was significantly lower than in asymptomatic patients. It is not clear whether the appearance of the symptoms is in a cause-effect relationship with altered cytokine production, but these data imply that cytokine determination might be a potential diagnostic tool, provided it predicts premature pregnancy termination with a higher precision than clinical symptoms. Because of the relatively small number of completed pregnancies, we are presently unable to answer this question.

Retrospective comparison of cytokine expression in lymphocytes of women whose pregnancies resulted in term labor with those of women whose pregnancies terminated prematurely revealed a significantly higher IL-10 expression and a slightly lower IL-12 expression in successful pregnancies.

The findings of this study, together with earlier data indicating that PIBF promotes the production of Th2 type

cytokines in vitro, suggest that during normal pregnancy PIBF might affect cytokine production.

Acknowledgments

This work was supported by grants from the Hungarian National Research Fund and the Hungarian Ministry of Welfare.

REFERENCES

1. Deanesly R: Termination of early pregnancy in rats after ovariectomy is due to immediate collapse of the progesterone dependent decidua. J Reprod Fertil 1973; 35:183–186.
2. Butterstein GM, Hirst JA: Serum progesterone and fetal morphology following ovariectomy and adrenalectomy in the pregnant rat. Biol Reprod 1977; 15:654–660.
3. Hansen PJ, Bazer FW, Segerson EC: Skin graft survival in the uterine lumen of ewes treated with progesterone. Am J Reprod Immunol Microbiol 1986; 12:48–54.
4. Szekeres J, Csernus V, Pejtsik B, Emody L, Pacsa AS: Progesterone as an immunologic blocking factor in human pregnancy serum. J Reprod Immunol 1981; 3:333–339.
5. Szekeres-Bartho J, Autran B, Debre P, Andreu G, Denver L, Chaouat G: Immunoregulatory effects of a suppressor factor from healthy pregnant women's lymphocytes after progesterone induction. Cell Immunol 1989; 122:281–294.
6. Szekeres-Bartho J, Kilar F, Falkay G, Csernus V, Torok A, Pacsa AS: Progesterone-treated lymphocytes of healthy pregnant women release a factor inhibiting cytotoxicity and prostaglandin synthesis. Am J Reprod Immunol Microbiol 1985; 5:15–18.
7. Szekeres-Bartho J, Szekeres Gy, Debre P, Autran B, Chaouat G: Reactivity of lymphocytes to a progesterone receptor-specific monoclonal antibody. Cell Immunol 1990; 125:273–281.
8. Szekeres-Bartho J, Faust Zs, Varga P: Progesterone-induced blocking factor (PIBF) in normal and pathological pregnancy. Am J Reprod Immunol 1995; 34:342–348.
9. Gendron R, Baines M: Infiltrating decidual natural killer cells are associated with spontaneous abortions in mice. Cell Immunol 1988; 113:261–268.
10. De Fougerolles R, Baines M: Modulation of natural killer activity influencesresorption rates in CBAxDBA/2 matings. J Reprod Immunol 1987; 11:147–153.
11. Kinsky R, Delage G, Rosin N, Thang MN, Hoffmann M, Chaouat G: A murine model for NK-mediated resorption Am J Reprod Immunol 1990; 23:73–77.
12. Szekeres-Bartho J, Kinsky R, Chaouat G: The effect of a progesterone induced immunologic blocking factor on NK mediated resorption. Am J Reprod Immunol 1990; 24:105–107.
13. Szekeres-Bartho J, Varga P, Pacsa AS: Immunological factors contributing to the initiation of labour - lymphocyte reactivity in term labour and threatened pre-term delivery. Am J Obst Gynecol 1986; 155:108–113.
14. Naume B, Espevik T: Immunoregulatory effects of cytokines on natural killer cells. Scand J Immunol 1994; 40:128–134.
15. Luft BJ, Remington JS: Effect of pregnancy on augmentation of natural killer cell activity be Corynebacterium parvum and Toxoplasma gondii. J Immunol 1984; 132:2375–2380.
16. Dresser DE: The potentiating effect of pregnancy on humoral immune responses of mice. J Reprod Immunol 1991; 20:253–266.
17. Paavonen T, Andersson LC, Adlercreutz H: Sex hormone regulation of in vitro immune response. Estradiol enhances human B cell maturation via inhibition of suppressor T cells in pokeweed mitogen-stimulated cultures. J Exp Med 1981; 154:1935–1945.
18. Lin H, Mosmann TR, Guilbert L, Tuntipopipat S, Wegmann TG: Synthesis of T helper 2-type cytokines at the maternal-fetal interface. J Immunol 1993; 151:4562–4567.
19. Szekeres-Bartho J, Wegmann G: A progesterone-dependent immunomodulatory protein alters the Th1/Th2 balance. J Reprod Immunol 1996; 31:81–95.
20. Szekeres-Bartho J, Par G, Dombay Gy, Smart Y, Volgyi Z: The anti-abortive effect of PIBF in mice is manifested by modulating NK activity. Cell Immunol: in press.
21. Wegmann TG, Hui L, Guilbert L, Mosmann TR: Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a Th2 phenomenon? Immunol Today 1993; 14:353–356.
22. Tangri S, Wegmann, TG, Lin H, Raghupathy R: Maternal anti-placental reactivity in natural immunologically-mediated fetal resorptions. J Immunol 1994; 152:4903–4911.
23. Chaouat G, Assal Meliani A, Martal J, et al: IL-10 prevents naturally occurring fetal loss in the CBAxDBA/2 mating combination and local defect in IL-10 production in this abortion-prone combination is corrected by in vivo injection of IFN-tau. J Immunol 1995; 154:4261–4268.

PAPER 2.

AJRI 1997; 38:176–182
Printed in the United States of America - all rights reserved.

American Journal of Reproductive Immunology
ISSN 8755-8920

Progesterone and Non-specific Immunologic Mechanisms in Pregnancy

JULIA SZEKERES-BARTHO, G. PAR, L. SZEREDAY, C.Y. SMART, AND I. ACHATZ

Szekeres-Bartho J, Par G, Szereday L, Smart CY, Achatz I. Progesterone and non-specific immunologic mechanisms in pregnancy. AJRI 1998; 37:176–182 © Munksgaard, Copenhagen

PROBLEM: Progesterone-dependent immunomodulation is one of the mechanisms that enables pregnancy to proceed to term. Immunologic effects of progesterone are mediated by a protein named the progesterone-induced blocking factor (PIBF). Among other effects this protein inhibits natural killer (NK) activity and displays an anti-abortive effect in mice. Recently we have shown that PIBF induces a Th2 shift *in vitro*. The present study was aimed at investigating the *in vivo* effect of PIBF on cytokine production, as well as the relationship between cytokine production, NK activity, and pregnancy loss.

METHOD OF STUDY: Balb-c mice on day 8.5 of pregnancy were injected intraperitoneally with 0.5 mg of rabbit anti-PIBF immunoglobulin G (IgG). Another group of mice was simultaneously treated with anti-NK monoclonal antibodies. Mice treated with the same amount of normal rabbit serum or untreated mice of similar gestational age were used as controls. The animals were sacrificed on day 10.5, and their uteri were inspected. The ratio of living and resorbed embryos was determined. NK activity as well as cytokine expression on the spleen cells were determined by immunocytochemistry and enzyme-linked immunoadsorbent assay (ELISA).

RESULTS: Mitogen-activated spleen cells from anti-PIBF-treated mice produced significantly ($P < 0.001$) less IL-10 than those of pregnant control mice. A significantly higher percentage ($P < 0.001$) of spleen cells from anti-PIBF-treated mice expressed interferon-γ (IFNγ) as determined by immunocytochemistry, than those of untreated pregnant mice. There was a positive relationship between the percentage of IFNγ-positive spleen cells and resorption rates, and an inverse relationship between the latter and interleukin-10 (IL-10) production. All these effects were corrected by treatment with anti-NK antibodies.

CONCLUSION: Our data suggest that PIBF contributes to the success of gestation via cytokine-mediated inhibition of NK activity.

Key words:
Cytokine, pregnancy, progesterone

JULIA SZEKERES-BARTHO
G. PAR
L. SZEREDAY
I. ACHATZ
Department of Microbiology
University Medical School of Pecs,
H-7643, Pecs, Hungary

C.Y. SMART
Discipline of Surgical Sciences,
Faculty of the Medical University
of Newcastle, Newcastle, Australia

Address reprint requests to Julia Szekeres-Bartho, Department of Microbiology, University Medical School of Pecs, H-7643 Pecs, Hungary

Submitted December 11, 1996;
accepted February 7, 1997.

INTRODUCTION

Many observations suggest that cell-mediated immunity is altered during pregnancy. The incidence of certain infections is increased,[1,2] malignancies are more frequent,[3,4] and the survival of skin homografts is prolonged in pregnant women.[5]

A long line of evidence supports the role of nonspecific immunologic mechanisms in pregnancy loss both in mice and humans. Natural killer (NK) activity is decreased in human pregnancy, whereas spontaneous pregnancy termination is associated with increased NK activity.[6] There is direct evidence for the role of high NK activity in pregnancy termination in mice. Resorption sites in mice are infil-

trated by NK cells.[7] Modulation of NK activity influences resorption rates.[8] Natural killer cells are recruited into the pregnant uterus during early pregnancy in several species,[9-11] however, their role is controversial. Rukavina et al.[12] demonstrated CD16$^-$, perforin$^+$, yet non-cytotoxic NK cells in first-trimester human decidua. Chao et al.[13] have shown that although the relative proportion of decidual NK cells is increased, decidual NK activity is lower in normal than in anembryonic pregnancies and in recurrent spontaneous abortions.

Nonspecific immunologic mechanisms including the action of NK and natural cytotoxic (NC) cells are probably the most important effector pathways in the feto-maternal immunologic relationship. NK activity in the decidua of pregnant mice is mediated in part by NC cells.[14] The immunologic effects of progesterone are mediated by a 34-kDa protein named the progesterone-induced blocking factor (PIBF).[15] Among other effects this protein inhibits NK activity[16] and displays an anti-abortive effect in mice.[17]

Normal pregnancy is characterized by decreased cell-mediated responses[18] and an increased rate of antibody production,[19] indicating a Th2-biased immune response during pregnancy. Strong cellular anti-fetal response is deleterious for pregnancy, whereas increased antibody production is not harmful.[20,21] In experimental conditions the outcome of pregnancy can be influenced by modulating the cytokine balance. The administration of tumor necrosis factor α (TNF-α), interferon (IFN-γ) or interleukin-2 (IL-2) to normal pregnant mice causes abortions,[22,23] whereas low doses of granulocyte macrophage colony-stimulating factor (GM-CSF), IL-3, or anti-TNF-α reduce resorption rates in a murine abortion model.[24,25] Th2-type cytokines IL-4, IL-5, and IL-10 are detectable in murine feto-placental units in all three trimesters of pregnancy.[26]

Recently we have shown that PIBF alters the profile of cytokine secretion by activated lymphocytes.[27] In our hands IL-3, IL-4, and IL-10 production of ConA-activated murine spleen cells was significantly increased in the presence of PIBF. Progesterone has been shown to exert a positive effect on the induction of IL-5 gene expression in T cell lines.[28]

The present study was aimed at investigating the *in vivo* effect of PIBF on cytokine production as well as the relationship between altered cytokine production, NK activity, and pregnancy loss in anti-PIBF treated mice.

MATERIALS AND METHODS

Treatment of Pregnant Mice by Anti-PIBF and Anti-NK Antibodies

Balb-c mice (from Lati, Godollo, Hungary) that were 8.5 days pregnant were injected intraperitoneally with 0.5 mg of rabbit anti-PIBF IgG. Mice treated with the same amount of normal rabbit serum or untreated mice of similar gestational age were used as controls. A group of anti-PIBF-treated mice was at the same time injected with monoclonal antibodies neutralizing NK or NC activity. The animals were sacrificed on day 10.5, and their uteri were inspected. The ratio of living and resorbed embryos was determined.

Spleens were removed under sterile conditions and single-cell suspensions were prepared. NK activity of the spleen cells was determined in a 16-hr cytotoxicity test.

The cell count was adjusted to 1×10^6, and the cells were cytocentrifuged on glass microscope slides. The slides were fixed in cold acetone, and the expression of IFN-γ and IL10 as well as that of PIBF on the lymphocytes was determined by immunocytochemistry.

Spleen cells were activated with ConA for 48 hr, then the supernatants were collected and IL-10 as well as IFN-γ were determined by enzyme-linked immunoadsorbent assay (ELISA).

Monoclonal Antibodies

The monoclonal 1C4 (anti-NC-1.1, mouse IgG1) was produced in our laboratory and was used in this study as an affinity purified preparation.[29] PK136 (anti-NK1.1 mouse IgG2b)[30] was obtained from the American Tissue Type Collection and was used as a serum-free supernatant.

The MoAb 2B6-F2 (anti-NK and anti-NC, rat IgG2a)[31] was produced in our laboratory and was used as a serum free supernatant.

Production of PIBF

Spleen cells of 10-week-old Balb/c mice (LATI, Godollo, Hungary) were adjusted to a cell count of 1×10^6/ml in RPMI supplemented with 10% fetal calf serum (FCS; both from Gibco, Grand Island, NY) and were stimulated by 1 μg/ml of ConA (Sigma, St. Louis, MO) for 48 hr at 37°C in a CO_2 incubator. The cell count was then adjusted to 10×10^6/ml, and the cells were further incubated with 20 μg/ml of progesterone for 16 hr. At the end of the incubation the supernatants were collected. Progesterone was removed by dialysis. The supernatants were then concentrated 2,000-fold on Amicon filters and were used as the source of the murine PIBF.

Production of PIBF-Specific IgG

Spleen cells of pregnant mice were treated with 20 μg/ml of progesterone overnight. The supernatants were collected, concentrated on Amicon filters, and subjected to polyacrilamide gel electrophoresis (PAGE) on 12% polyacrylamide gels. The separated bands were blotted to nitrocellulose filters, the 34-kDa band was cut out, dissolved in DMSO and injected to rabbits weighing 4 kg each together with complete Freund adjuvant. Boosters with incomplete Freund adjuvant were given at 2-week intervals. Immunoglobulin G (IgG) was purified on protein A columns.

Immunocytochemical Determination of Cytokines

Spleen cells were washed once, the cell count was adjusted to 1 × 10^6/ml, and the cells were centrifuged on glass microscope slides.

After drying at room temperature, the cells were fixed for 5 min in cold acetone and were washed with phosphate-buffered saline (PBS).

All incubations were performed at room temperature in a humidified atmosphere. Anti-IL-10 and anti-IFN-γ monoclonal antibodies (both from Endogen, Cambridge, MA) were applied at a dilution of 1:50, and the 2nd antibody (peroxidase labeled anti-rat antibody) was used at a dilution of 1:100. The reaction was developed by aminoetylcarbasol, nuclei were counterstained with haematoxylin, and slides were mounted with gelatine-glycerol. The percentage of positive cells was determined by light microscopy, counting 300 cells.

Cytotoxicity Assay

This technique has been described elsewhere in detail.[32] Briefly, human embryonic fibroblasts derived from 10- to 12-week embryos were used as targets. Cells were seeded on 96-well Nunclon tissue culture plates at a density of 5,000 target cells/well in 0.2 ml of medium 199 supplemented with 10% FCS. The target cells were allowed to attach by overnight incubation. The following day the medium was replaced with 0.2 ml of lymphocyte suspension containing 5 × 10^5 lymphocytes in 0.2 ml of the same medium. After 16-hr of incubation, the plates were washed with PBS three times to remove lymphocytes and damaged target cells. Then the substrate of alkaline phosphatase (Sigma tablets No. 104) was added to the wells in diethanolamine buffer at a concentration of 1 mg/ml. The plates were incubated for 10 min at 37°C in the dark, and the resulting yellow reaction product was quantitated photometrically at 405 nm. The percentage reduction in enzyme activity relative to the target cell control was considered as a measure of cytotoxicity.

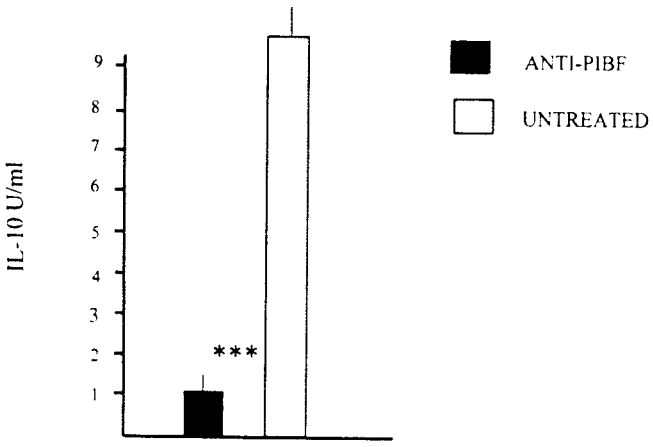


Fig. 1. Anti-PIBF treatment inhibits splenic IL-10 production. The bars represent the mean ± SEM. ***$P < 0.001$.

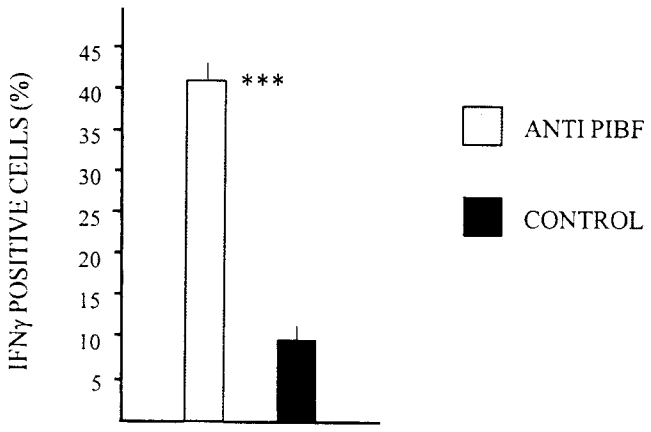


Fig. 2. The effect of anti-PIBF treatment on splenic IFN-γ production. The bars represent the mean ± SEM. ***$P < 0.001$.

Determination of Cytokines by ELISA

Spleen cells from treated and untreated pregnant Balb/c mice were washed and, the cell count was adjusted to 1 × 10^6/ml. The cells were activated with 1 μg/ml of ConA (Sigma) for 48 hr. After 48 hr the supernatants were collected and tested for IL-10. For IL-10 determination we used a Biotrak (Amersham, Little Chalfont, UK) kit. The assay was performed following the steps suggested by the manufacturer.

Statistical Analysis

The two-tailed Student's t test and the χ^2 test were used for statistical analysis of the data. Mean ± SEM are indicated in the tables and figures. Differences were considered to be significant at $P < 0.05$.

RESULTS

Inhibition of Progesterone-Dependent Immunomodulation Results in Altered Cytokine Production

On day 8.5 of pregnancy Balb/c mice were treated with 0.5 mg of anti-PIBF IgG. On day 10.5 the animals were sacrificed and splenic cytokine production was determined by ELISA and immunocytochemistry. In mice treated with anti-PIBF on day 8.5 of pregnancy, splenic IL-10 production was significantly lower ($P < 0.001$) than that in untreated controls (Fig. 1).

The treatment resulted in an increased percentage of IFN-γ-positive spleen cells (Fig. 2). There was a positive

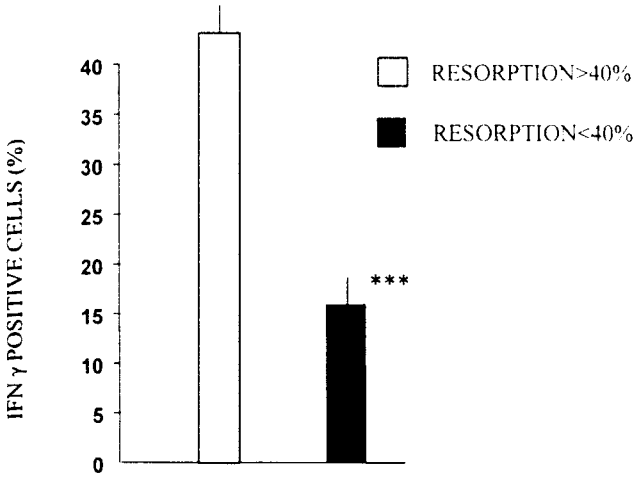


Fig. 3. The relationship between splenic IFN-γ expression and resorption rates. The bars represent the mean ± SEM. ***$P < 0.001$.

relationship between NK activity and the percentage of IFN-γ-positive spleen cells. Furthermore, in animals with a high resorption rate the rate of splenic IFN-γ expression was significantly ($P < 0.001$) higher than in those with a low resorption rate (Fig. 3).

Splenic IL-10 production, on the other hand, was inversely related to resorption rates (Fig. 4).

IL-10 and IFN-γ expression on spleen cells of anti-NK treated mice

A group of anti-PIBF-treated mice was injected with anti-NK or anti-NC antibodies. This treatment resulted in a significant reduction ($P < 0.001$) in the percentage of IFN-γ+ spleen cells (Fig. 5). The highest inhibition in the ratio of IFN-γ+ spleen cells was obtained using the antibody 2B6-F2, which neutralizes both NK and NC activity (Fig. 6).

Anti-NK treatment corrected the decreased splenic IL-10 production in anti-PIBF-treated mice from 3 to 9.93 mU/ml (Fig. 7).

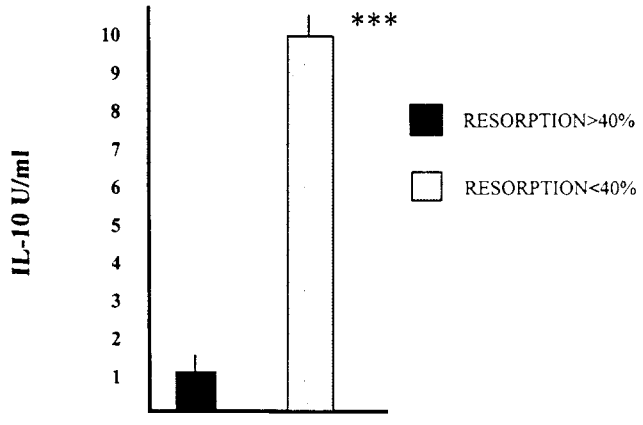


Fig. 4. The relationship between IL-10 production by spleen cells and resorption rates. The bars represent the mean ± SEM. ***$P < 0.001$.

DISCUSSION

Cytokines are indispensable molecules for information processing within the immune system. Concerning their effect on the immune response they can be divided into two categories. Th1 cytokines increase cell-mediated responses, whereas Th2 cytokines increase humoral responsiveness and inhibit cell-mediated responses.

Pregnancy is a Th2-like phenomenon. During normal pregnancy immunoglobulin synthesis is increased,[18] whereas cell-mediated responses (especially NK activity) are decreased.[19] In mice, predominantly Th2-type cytokines

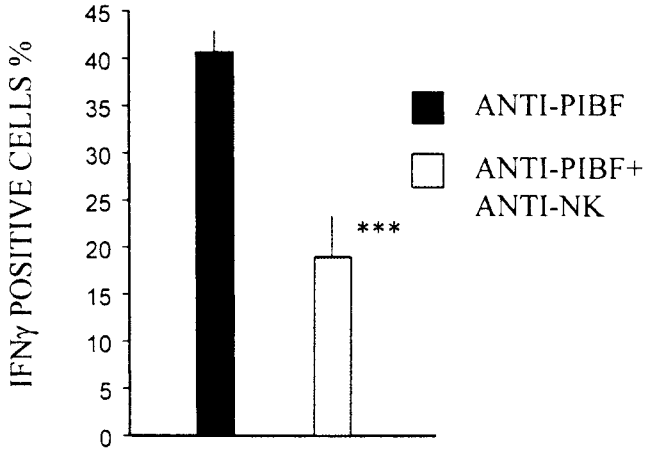


Fig. 5. IFN-γ expression on spleen cells from pregnant mice treated with anti-NK monoclonal antibodies after neutralization of endogenous PIBF activity. The bars represent the mean ± SEM. ***$P < 0.001$.

IL-4, IL-5, and IL-10 are detectable in the feto-placental units throughout gestation.[26]

A long line of observations suggests the association of NK activity and pregnancy termination in mice.[7,8] In human pregnancy we demonstrated a significant increase in NK activity before term.[6] Because PIBF normally blocks NK activity, the lack of this protein would result in increased NK cell function. Our previous data revealed an association of pregnancy termination and the lack of lymphocytic PIBF positivity, as well as a negative correlation between PIBF production and NK activity.[32] Thus, PIBF determines the relationship between pregnancy termination and increased NK activity. The mechanism through which PIBF influences NK activity is manifested via multiple systems, and the relationship between the different pathways has not been clarified yet:

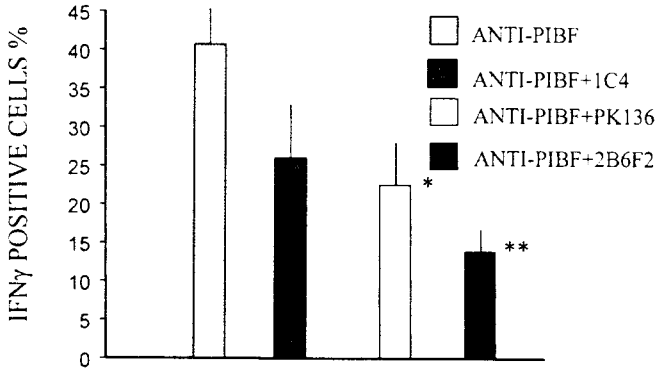


Fig. 6. IFN-γ production of spleen cells from pregnant mice treated with anti-NK and anti-NC monoclonal antibodies after neutralization of endogenous PIBF activity. The bars represent the mean ± SEM. $^{***}P < 0.001$.

- Via inhibiting the release of arachidonic acid, PIBF reduces prostaglandin and leukotriene production.[15]

- In the presence of PIBF, B cells produce hypermannosilated antibodies,[33] and inhibition of high mannose-type glycosilation results in increased NK activity (manuscript in preparation).

- PIBF affects cytokine secretion of *in vitro* activated lymphocytes.[32] Its action on NK activity might involve cytokine effect, because though the trophoblast resists NK-mediated lysis *in vitro*, it is susceptible to lysis by lymphokine-activated killer (LAK) cells.[34] NK-like cells may cause a fetal loss by being converted in the presence of TNF-α and IL-2 into LAK cells.[35] PIBF-producing pregnancy lymphocytes display a low NK activity.[36] In *in vitro* systems we have shown that increased NK activity of pregnancy lymphocytes due to neutralization of PIBF by a specific antibody is corrected by simultaneous administration of IL-10 or anti-IL-12.[37] In line with this observation, mitogen-activated lymphocytes of nonpregnant individuals produce less IL-12 in the presence than in the absence of PIBF.[37] In pregnant mice, the neutralization of endogenous PIBF activity resulted in a high resorption rate and NK activity, together with decreased splenic IL-10 and increased IFN-γ production. Because IL-10 inhibits cytokine production by Th1-type cells as well as CD8+ T cells,[38] the lack of IL-10 production might be one of the factors responsible for high NK activity.

The neutralization of NK and NC activity in anti-PIBF-treated mice corrected not only resorption rates but also cytokine values. This implies that NK cells are not simply targets of cytokines, thus the abortive effect of anti-PIBF treatment is not merely due to LAK cells, but these cells are producers of the cytokines themselves. In the mouse IL-10 is produced by Th2 cells as well as by macrophages and B cells.[39,40] IFN-γ is produced by NK cells. Clark and Chaouat[41] have shown that not NK activity per se, but cytokines produced by the NK cells are responsible for the high resorption rates in mice. Treatment of pregnant CBA/J mice with anti-asialoGM1 antibody lowered the background abortion rate to 10% or less, and the rate of abortions could not be restored by injecting TNF-α or IFN-γ alone. However, both cytokines together led to >80% rate of abortions, much higher than that expected, based on the implantation sites showing excessive macrophage/NK infiltration.

Recently we have shown that treatment of anti-PIBF-treated mice with anti-NK or anti NC monoclonal antibodies corrected the high resorption rates and the low mean implantation sites (manuscript in preparation).

In the present experiments injection of anti-PIBF-treated mice with anti-NK and anti-NC monoclonal antibodies corrected the high splenic IFN-γ, and IL-10 production. The PK136 antibody reacts with LGL-1-cells. This is a functional subset of NK-1.1$^+$ cells that contains the majority of LAK cell progenitors.[42] The trophoblast is resistant to lysis by NK cells, but it is lysed by LAK cells,[34] thus the latter cell type may be involved in trophoblast damage. The antibody 1C4 reacts with natural cytotoxic cells, and the third monoclonal antibody (2B6-F2) reduces both NK and NC activity in Balb/c mice.

Our data suggest that PIBF contributes to the success of gestation via cytokine-mediated inhibition of NK activity.

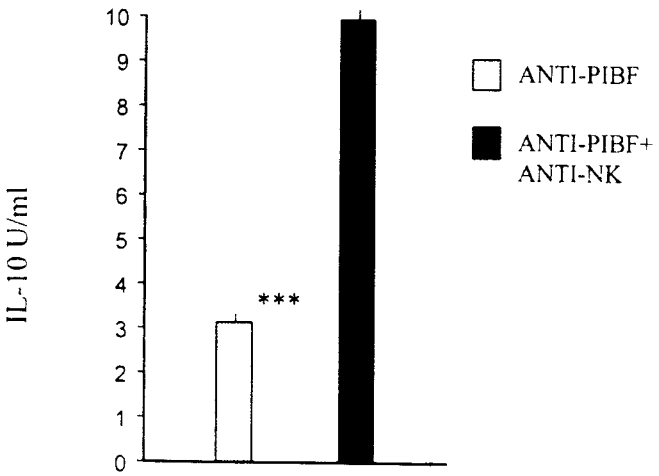


Fig. 7. IL-10 production of spleen cells from pregnant mice treated with anti-NK monoclonal antibodies after neutralization of endogenous PIBF activity. The bars represent the mean ± SEM of determinations. $^{*}P < 0.001$.

REFERENCES

1. Pickard RE: Varicella pneumonia in pregnancy. Am J Obstet Gynecol 1968; 100:504–508.
2. Vaughan JE, Ramirez H: Coccidioidmycosis as a complication of pregnancy. Calif Med 1951; 74:121–125.
3. Gleicher N, Cohen CJ, Deppe G: Familial malignant melanoma of the female genitalia. Obstet Gynecol Survey 1979; 34:1–15.
4. Janerich DT: The influence of pregnancy on breast cancer risk. Is it endocrinological, or immunological? Med Hypotheses 1980; 6:1149–1155.
5. Andresen RH, Monroe CW: Experimental study of the behavior of adult human skin homografts during pregnancy. Am J Obstet Gynecol 1962; 84:1096–1103.
6. Szekeres-Bartho J, Varga P, Pacsa AS: Immunological factors contributing to the initiation of labour-lymphocyte reactivity in term labour and threatened pre-term delivery. Am J Obstet Gynecol 1986; 155:108–113.
7. Gendron R, Baines M: Infiltrating decidual natural killer cells are associated with spontaneous abortions in mice. Cell Immunol 1988; 113:261–268.
8. De Fougerolles R, Baines M: Modulation of natural killer activity influences resorption rates in CBA × DBA/2 matings. J Reprod Immunol 1987; 11:147–153.
9. Starkey PM, Sargent LL, Redman CWG: Cell populations in human early pregnancy decidua: Characterization and isolation of large granular lymphocytes by flow cytometry. Immunology 1988; 65:129–134.
10. Croy BA, Gambel P, Rossant J, Wegmann TG: Characterization of murine decidual natural killer (NK) cells and their relevance to the success of pregnancy. Cell Immunol 1985; 93:315–326.
11. Croy BA, Waterfield A, Wood W, King GJ: Normal murine and porcine embryos recruit NK cells to the uterus. Cell Immunol 1988; 115:471–480.
12. Rukavina D, Rubesa G, Gudelj L, Haller H, Podack E: Characteristics of perforin expressing lymphocytes within the first trimester decidua of human pregnancy. Am J Reprod Immunol 1995; 33:394–404.
13. Chao K-H, Yang Y-S, Ho H-N, Chen S-U, Chen H-F, Dai H-J, Huang S-C, Gill TJ III: Decidual natural killer cytotoxicity decreased in normal pregnancy but not in anembryonic pregnancy and recurrent spontaneous abortion. Am J Reprod Immunol 1995; 34:274–280.
14. Clarke GR, Roberts TK, Smart YC: Natural killer and natural cytotoxic cells are present at the maternal-fetal interface during murine pregnancy. Immunol Cell Biol 1994; 72:153–160.
15. Szekeres-Bartho J, Kilar F, Falkay G, Csernus V, Torok A, Pacsa AS: Progesterone-treated lymphocytes of healthy pregnant women release a factor inhibiting cytotoxicity and prostaglandin synthesis. Am J Reprod Immunol Microbiol 1985; 9:15–18.
16. Szekeres-Bartho J, Autran B, Debre P, Andreu G, Denver L, Chaouat G: Immunoregulatory effects of a suppressor factor from healthy pregnant women s lymphocytes after progesterone induction. Cell Immunol 1989; 122:281–294.
17. Szekeres-Bartho J, Chaouat G: Lymphocyte-derived progesterone induced blocking factor corrects resorption in a murine abortion system. Am J Reprod Immunol 1990; 23:26–28.
18. Luft BJ, Remington JS: Effect of pregnancy on augmentation of natural killer cell activity be *Corynebacterium parvum* and *Toxoplasma gondii*. J Immunol 1984; 132:2375–2380.
19. Dresser DE: The potentiating effect of pregnancy on humoral immune responses of mice. J Reprod Immunol 1991; 20:253–266.
20. Wegmann TG, Waters CA, Drell DW, Carlson GA: Pregnant mice are not primed but can be primed to fetal alloantigens. Proc Natl Acad Sci U S A 1979; 76:2410–2414.
21. Billington WD: Transfer of antigens and antibodies between mother and fetus. *In* Immunological Obstetrics, CB Coulam, WP Faulk, J McIntyre (eds). New York, W.W. Norton & Co., 1992, pp 290–304.
22. Parant M: The role of TNF in nonspecific stimulation of the mouse resistance to infection. Immunobiology 1987; 175:26.
23. Tezabwala BV, Johnstone AP: Effect of administration of interleukin 2 in pregnancy. J Reprod Immunol 1986; 1:(Suppl):147.
24. Hill JA: Implication of cytokines in male or female sterility. *In* Cellular and molecular biology of the feto-maternal relationship, G. Chaouat and JF Mowbray (eds). Paris, Institute Nationale de la Sante et de la Recherche Medicale, 1991, pp 123–130.
25. Chaouat G, Menu E, Wegmann TG: Role of lymphokines of the CSF family and of TNF, gamma-interferon and IL-2 on placental growth and fetal survival, studied in 2 murine models of spontaneous resorptions. *In* Cellular and Molecular Biol. of the Materno-Fetal Relationship, Vol. 212, G Chaouat, G Mowbry (eds). Paris, London, Colloque INSERM John Libbey Eurotext Ltd., 1991. 91–100.
26. Lin H, Mosmann TR, Guilbert L, Tuntipopipat S, Wegmann TG: Synthesis of T helper 2-type cytokines at the maternal-fetal interface. J Immunol 1993; 151:4562–4567.
27. Szekeres-Bartho J, Wegmann TG: A progesterone-dependent immunomodulatory protein alters the Th1/Th2 balance. J Reprod Immunol 1996; 31:81–95.
28. Wang Y, Campbell HD, Young IG: Sex hormones and dexamethasone modulate interleukin-5 gene expression in T lymphocytes. J Steroid Biochem Mol Biol 1993; 44:203–210.
29. Smart YC, Stevenson KL, Farrelly ML, Brien JH, Burton RC: Production of a monoclonal allo-antibody to murine natural cytotoxic cells. Immunol Cell Biol 1990; 68:277–284.
30. Koo GC, Peppard JR: Establishment of monoclonal anti NK-1.1 antibody. Hybridoma 1984; 3:301–303.
31. Martinello R, Smart YC, Burton RC: Monoclonal antibody 2B6-F2 identifies a subset of murine natural killer cells with receptors for YAC-1 and WEHI-164 targets. Cell Immunol 1994; 156:155–169.
32. Szekeres J, Pacsa AS, Pejtsik B: Measurement of lymphocyte cytotoxicity by assessing endogenous alkaline phosphatase activity of the target cells. J Immunol Methods 1981; 40:151–154.
33. Kelemen K, Bognar I, Szekeres-Bartho J: A progesterone induced protein increases the ratio of non-precipitating antibodies. Cell Immunol 1966; 167:129–134.
34. Drake BL, Head JR: Murine trophoblast cells can be killed by lymphokine-activated killer cells. J Immunol 1989; 143:9–14.
35. Clark DA: Are there immune abortions? Res Immunol 1990; 141:104–109.
36. Szekeres-Bartho J, Faust Zs, Varga P: Progesterone-induced blocking factor (PIBF) in normal and pathological pregnancy. Am J Reprod Immunol 1995; 34:342–345.
37. Szekeres-Bartho J, Faust Zs, Varga P, Szereday L, Kelemen K: The immunological pregnancy protective effect of

progesterone is manifested via controlling cytokine production. Am J Reprod Immunol 1996; 35:348–351.
38. Thompson-Snipes L, Dhar V, Bond MW, Mosmann TR, Moore KW, Rennick DM: Interleukin-10: A novel stimulatory factor for mast cells and their progenitors. J Exp Med 1991; 173:507–510.
39. LeGros G, Erard F: Non-cytotoxic, IL-4, IL-5, IL-10 producing CD8+ T cells: Their activation and effector functions. Curr Opin Immunol 1994; 6:453–457.
40. de Waal Malefyt R, Abrams J, Bennet B, Figdor CG, de Vries J: Interleukin 10 (IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. J Exp Med 1991; 174:1209–1220.
41. Clark DA, Chaouat G: Spontaneous abortion in DBA/2 mated mice is due to cytokines and not trophoblast killing by asialo GM1+ NK cells or NO-producing macrophages. Am J Reprod Immunol 1996; 35:454(Abstract).
42. Mason LH, Mathieson BJ, Ortaldo J: Natural Killer (NK) cells subsets in the mouse: NK-1.1$^+$/LGL-1$^+$ cells restricted to lysing NK targets, whereas NK-1.1$^+$/LGL-1$^-$ cells generate lymphokine activated killer cells. J Immunol 1990; 145:751–759.

PAPER 3.

AJRI 1996; 35:348–351
Printed in the United States of America - all rights reserved.

American Journal of Reproductive Immunology
ISSN 8755-8920

The Immunological Pregnancy Protective Effect of Progesterone Is Manifested via Controlling Cytokine Production

JULIA SZEKERES-BARTHO, ZS. FAUST, P. VARGA, L. SZEREDAY, AND K. KELEMEN

Szekeres-Bartho J, Faust Zs, Varga P, Szereday L, Kelemen K. The immunological pregnancy protective effect of progesterone is manifested via controlling cytokine production. AJRI 1996; 35:348–351 © Munksgaard, Copenhagen

PROBLEM: This study was aimed at investigating the involvement of an altered cytokine pattern in the immunomodulatory and anti-abortive effects of a progesterone-induced immunomodulatory protein (PIBF).

METHOD: PIBF expression on lymphocytes of healthy pregnant women and from women at risk for premature pregnancy termination was determined. In sera of the same women TNFα was quantified by a bioassay using L929 cells. NK activity was determined by a single cell cytotoxicity assay. Cytokine production of the lymphocytes or murine spleen cells was measured by ELISA or detected by immunocytochemistry. In pregnant mice endogenous PIBF activity was neutralized by anti-PIBF IgG.

RESULTS: Sera of women at risk for premature pregnancy termination contained significantly higher concentrations of TNFα than those from healthy pregnant women and PIBF expression on the lymphocytes was inversely related to serum concentration of TNFα. Increased NK activity of lymphocytes after neutralization of endogenous PIBF activity is corrected by anti-IL2 treatment and PIBF inhibits IL12 expression on activated lymphocytes. PIBF increases IL-10 production by activated spleen cells. In pregnant mice, neutralization of endogenous PIBF activity by specific antibody results in increased resorption rate and reduced splenic IL-10 production.

CONCLUSIONS: Our data allow the assumption that via blocking IL-12 production PIBF inhibits NK activation with a concomitant reduction of TNFα levels. Disturbances in this system might lead to the expression of the known synergistic effect of IL-12 and TNFα, resulting in a Th1 type cytokine dominance and pregnancy termination.

Key words:
Cytokines, progesterone, progesterone-induced immunomodulatory protein

JULIA SZEKERES-BARTHO
ZS. FAUST
P. VARGA
L. SZEREDAY
K. KELEMEN
Department of Microbiology, University Medical School of Pecs, Pecs, Hungary

Presented at the Sixth International Congress of Reproductive Immunology, Washington, DC, July 19–23, 1995.

Address reprint requests to J. Szekeres-Bartho, Department of Microbiology, University Medical School of Pecs, H-7643 Pecs, Hungary.

Submitted August 3, 1995; accepted October 10, 1995.

INTRODUCTION

In the presence of progesterone, lymphocytes of healthy pregnant women produce a 34 kDa immunomodulatory protein (PIBF).[1] PIBF inhibits NK activity and prevents resorptions in mice.[2]

Earlier we demonstrated a significant increase of NK activity in women showing clinical symptoms of threatened premature pregnancy termination.[2] It is conceivable that high NK activity in women at risk for premature pregnancy termination is due to impaired PIBF producing capacity of the lymphocytes.

Normal human pregnancy is characterized by a low NK activity and a Th2 shift.[3]

Since altered NK activity in pregnant women seems to be at least in part progesterone-mediated, we investigated the involvement of an altered cytokine balance in NK-modulating effect of PIBF.

MATERIALS AND METHODS

Patients

Lymphocytes of 103 healthy pregnant women and those of 62 women at risk for premature pregnancy termination were tested. Ten women were sampled at the onset of spontaneous abortion or preterm delivery. PIBF expression on the lymphocytes was determined by immunocytochemistry using PIBF-specific polyclonal antibody.

Cytokine production of the lymphocytes was measured by ELISA or detected by immunocytochemistry.

For determining IL-12 expression on activated lymphocytes, human lymphocytes from nonpregnant individuals were at a cell count of 1×10^6/ml activated with 1 μg/ml of PHA (Sigma) for 48 h either in the presence or in absence of 2 μg/ml PIBF. After incubation, the cells were washed once and centrifuged on glass microscope slides. After drying at room temperature, the cells were fixed for 5 min in cold acetone and washed in phosphate buffered saline (PBS). All incubations were performed at room temperature in a humidified atmosphere. The primary antibody (polyclonal anti-human IL-12) (R&D Systems, Abingdon, Oxon, UK) was applied in a concentration of 10 μg/ml for 1 h. Peroxidase labeled anti-rabbit IgG (Dakopatts, Hungary) was used in a dilution of 1:200 for 45 min. The reaction was developed by aminoetylcarbasol, nuclei were counterstained with hematoxylin and slides were mounted with gelatine-glycerol. The percentage of positive cells was determined by light microscopy counting 300 cells.

IL-10 production of murine spleen cells was determined by ELISA, using an Amersham Biotrak system. The assay was performed according to the suggestions of the manufacturer.

TNFα was quantified by a bioassay using L929 cells.

Neutralization of endogenous PIBF activity was achieved by treating mice on day 8.5 of pregnancy with 0.5 mg of rabbit anti-PIBF IgG. On day 10.5, anti-PIBF treated and control pregnant animals were sacrificed, the rate of resorptions was recorded and spleens were removed aseptically. Spleen cells were isolated and at a cell count of 1×10^6/ml activated with 1 μg/ml of ConA (Sigma) for 48 h. At the end of the incubation supernatants were collected and IL-10 concentrations were determined by ELISA

Statistical analysis of the data was done by the two tailed Student's t-test.

RESULTS

The Relationship Between Serum TNFα Concentration and PIBF Expression on Lymphocytes

The concentration of TNFα in human serum was determined by measuring cytotoxicity to L929 cells. Sera of women at risk for premature pregnancy termination contained a significantly higher concentration of than of healthy pregnant women and PIBF expression on the lymphocytes was inversely related to serum concentration of TNFα (Fig.1).

The Effect of PIBF on NK Activity is Mediated by Cytokines

PIBF-producing pregnancy lymphocytes display a low NK activity. Neutralization of PIBF by a specific antibody results in increased NK activity, and this is corrected by simultaneous administration of IL-10. Given that addition of exogenous PIBF is able to overcome the NK stimulating effect of anti IL-10, PIBF possibly uses pathways other than the IL-10 pathway for inhibiting NK activity. Its effect might rather be mediated by inhibiting IL-12 synthesis, as suggested by the finding that anti IL-12 treatment inhibits increased NK-mediated lysis due to anti-PIBF treatment (Table 1).

In line with this observation, mitogen activated lymphocytes of non-pregnant individuals produce less IL-12 in the presence than in absence of PIBF (Fig. 2).

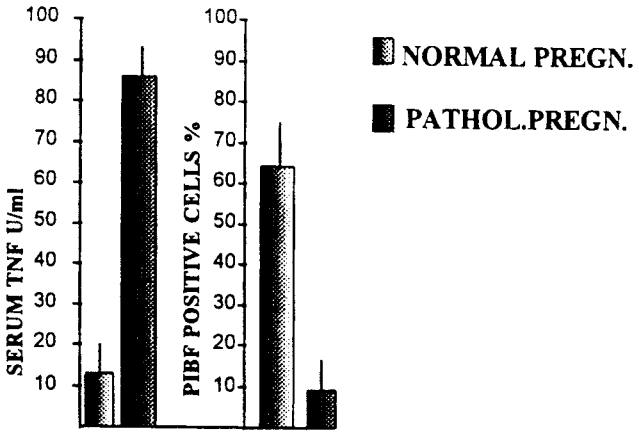


Fig. 1. The relationship between serum concentration and PIBF expression on the lymphocytes in pregnancy. The bars represent the mean ± SEM of 103 (normal pregnancy) 62 (pathological pregnancy) determinations. Serum levels were significantly higher ($P<0.001$), the ratio of PIBF+ lymphocytes was significantly lower ($P<0.001$) in pathological than in normal pregnancies.

TABLE I. The Effect of PIBF on NK Activity Is Mediated by Cytokines

Treatment of lymphocytes	NK activity % (mean±SEM)
None	1.7 ± 0.2
Anti-PIBF	3.6 ± 0.34***
Anti-PIBF+IL-10	1.0 ± 0.08**
Anti IL-10	3.0 ± 0.28***
Anti IL-10+PIBF	1.0 ± 0.2*
Anti-PIBF+anti IL12	0.6 ± 0.08****

**Significantly different at* $P<0.05$.
***Significantly different at* $P<0.02$.
****Significantly different at* $P<0.01$.
*****Significantly different at* $P<.0.001$

Neutralization of Endogenous PIBF Activity in Pregnant Mice Results in Altered Cytokine Production

Recent data from our laboratory revealed an increased IL-10 production by activated murine spleen cells in the presence of PIBF.[4] In day 8.5, pregnant mice neutralization of endogenous PIBF activity by anti-PIBF IgG results in increased resorption rate and reduced splenic IL-10 production (Table II).

DISCUSSION

Cytokines have been shown to affect pregnancy outcome.[5] It is well established that a strong cellular immune response is deleterious for pregnancy.[6,7] NK activity seems to play a role in spontaneous pregnancy termination.[6] PIBF inhibits NK activity.[8] Our present data suggest that this effect is cytokine-mediated. Because activated lymphocytes produced increased amounts of IL-10 in the presence of PIBF, it seemed conceivable that PIBF inhibited NK activity by increasing IL-10 synthesis. Both anti-PIBF and anti L-10 treatments increased NK activity. Exogenous IL-10 corrected the effect of anti-PIBF on NK activity. PIBF was also able to compensate for the NK stimulatory effect of anti IL-10 treatment, suggesting that the NK inhibitory effect of PIBF is not mediated solely by increasing IL-10 synthesis and release. This concept is supported by the findings that after neutralization of endogenous PIBF in vitro, IL-12 neutralizing antibody alone, prevents increase of NK activity and that PIBF inhibits IL-12 synthesis by activated lymphocytes. Recent data from our laboratory revealed an increased IL-12 production by peripheral lymphocytes of women with pathological pregnancies and high NK activity (manuscript in preparation).

Sera of women at risk for premature pregnancy termination contained significantly higher concentrations of TNFα than those from healthy pregnant women and PIBF expression on the lymphocytes was inversely related to serum concentration of TNFα.

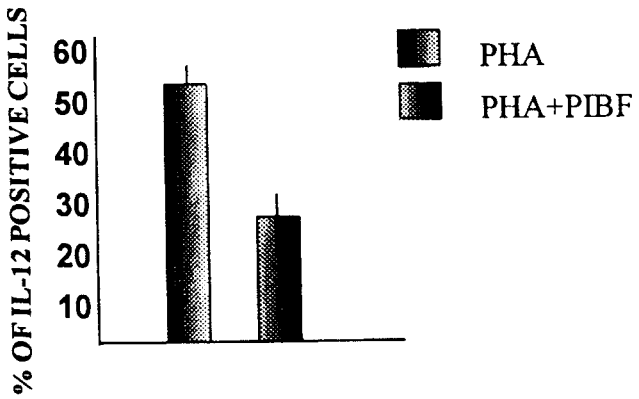


Fig. 2. The effect of PIBF on IL-12 expression of mitogen-activated human peripheral lymphocytes. The bars represent the mean ± SEM of 10 determinations. The two groups are significantly different ($P<0.01$).

TABLE II. Neutralization of Endogenous PIBF Activity in Pregnant Mice Results in Altered Cytokine Production

Treatment	IL-10(U/ml) mean ± SEM	Resorption rate % mean ± SEM
None (n=20)	10.0 ± 1.2	10 ± 0.5
Anti-PIBF (n=20)	1.0 ± 0.08*	60 ± 7.5*

**Significantly different at* $P<0.01$.

Earlier we demonstrated increased IL-10 production by activated murine spleen cells in the presence of PIBF.[4] In vivo experiments provided supportive evidence for the cytokine-mediated anti-abortive effect of PIBF. Neutralization of endogenous PIBF in pregnant mice resulted in increased resorption rate, together with high NK activity and reduced splenic IL-10 production. Based on these data we hypothesize that via increasing IL-10 and IL-12 production, PIBF inhibits NK activation. Disturbances in this system might lead to a Th1 type cytokine dominance and pregnancy termination.

Acknowledgments
This work was supported by grants from the Hungarian Ministry of Welfare and the Hungarian National Research fund.

REFERENCES

1. Szekeres-Bartho J, Kilar F, Falkay G, Csernus V, Torok A, Pacsa AS. Progesterone-treated lymphocytes of healthy pregnant women release a factor inhibiting cytotoxicity and prostaglandin synthesis. Am J Reprod Immunol Microbiol 1985; 9:15–18.

2. Szekeres-Bartho J. Immunosuppression by progesterone in pregnancy. Monograph. CRC Press, Boca Raton, CA, 1992.
3. Wegmann TG, Hui Lin, Guilbert L, Mosmann TR. Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a Th2 phenomenon? Immunology Today 1993; 14:353–356.
4. Szekeres-Bartho J, Wegmann TG. A progesterone-dependent immunomodulatory protein alters the Th1/Th2 balance (abstract) p. 313. 12th European Immunology Meeting 1994.
5. Clark DA. Are there immune abortions? Res Immunol 1990; 141:202–207.
6. De Fougerolles R, Baines, M. Modulation of natural killer activity influences resorption rates in CBAxDBA/2 matings. J Reprod Immunol 1987; 11:147–153.
7. Gendron R, Baines M. Infiltrating decidual natural killer cells are associated with spontaneous abortions in mice. Cell Immunol 1988; 113:261–268.
8. Szekeres-Bartho J, Autran B, Debre P, Andreu G, Denver L, Chaouat G. Immunoregulatory effects of a suppressor factor from healthy pregnant women's lymphocytes after progesterone induction. Cell Immunol 1989; 122:281–290.

PAPER 4.

NK ACTIVITY AND CYTOKINE PRODUCTION OF PREGNANCY LYMPHOCYTES AFTER IN VITRO IMMUNOGLOBULIN TREATMENT

L. Szereday , P. Späth*, J. Szekeres-Bartho
Department of Microbiology, University Medical School of Pecs, Hungary.
*ZLB Central Laboratory, Blood Transfusion Service SRC, Bern, Switzerland

Correspondence to: Julia Szekeres-Bartho M.D., Ph.D.
Department of Microbiology
University Medical School of Pecs
H-7643 Pecs, Hungary
Fax: 36 (72) 315 799
E-mail: szjuli@main.pote.hu

Acknowledgements
This work was supported by grants from the Hungarian National Research Fund and Hungarian Ministry of Welfare and the Ministry of Education

ABSTRACT

Problem To investigate the mechanism of immunoglobulin treatment in RSA, we determined the effect of in vitro immunoglobulin treatment on the rate of IL-10 and IL-12 positive cells among peripheral lymphocytes of healthy pregnant women and women at risk for premature pregnancy termination, including primary habitual aborters as well as women showing clinical symptoms (bleeding or regular uterine contractions) of threatened premature pregnancy termination.

Methods: Lymphocytes of 20 pregnant women were tested. Immunoglobulin preparations were obtained from Sandoz. Cytokine profile of the lymphocytes were determined by immunocytochemistry, for testing NK activity we used the 4 h single cell cytotoxicity assay.

Results: Incubation with immunoglobulin preparations significantly ($p<0.01$) decreased the rate of IL-12 positive cells and at the same time significantly ($p<0.01$) increased the rate of IL-10 positive cells among lymphocytes of recurrent spontaneous aborters, whereas the treatment had no significant effect on lymphocytes of healthy pregnant women

Dialysis or heat treatment (56°C, 30 min) of the immunoglobulin preparations did not modify the effect.

High NK activity of women at risk for premature pregnancy termination significantly decreased after immunoglobulin treatment in all cases, whereas NK activity of normal pregnancy lymphocytes was not altered.

INTRODUCTION

An increasing amount of evidence suggests that, the majority of recurrent spontaneous abortions (RSA) has an immunologic background.

Previous concepts proposing that RSA occurs due to a failure of the mother to produce blocking antibodies against paternal human lymphocyte antigens or HLA-linked antigens expressed on the fetus or trophoblast, have not gained sufficient evidence. [1,2]

Recent data suggest that increased cytotoxicity of decidual or peripheral blood natural killer cells against fetal antigens plays a role in the development of RSA.[3,4]

Normal pregnancy is characterised by a predominantly Th-2 type response against trophoblast antigens [5], whereas RSA in the majority of cases is characterised by a Th-1 response.[6]

Intravenous immunoglobulin treatment of recurrent aborters has been widely used as an alternative, for leukocyte immunisation.[7] However, the mechanism by which Ivlg therapy would prevent abortion has not been clarified.

Whatever the precise pathophysiologic mechanisms of immunologically mediated RSA are, there is a good chance that the infusion of intravenous immunoglobulin (Ivlg) would be beneficial. Since Ivlg is prepared from thousands of healthy donors, including multiparous women, passive infusion of blocking antibodies was presumed to be responsible for the beneficial effect observed from Ivlg.

The effects of Ivlg treatment might involve an increased elimination of circulating immune complexes. Inhibition of binding of the antibody or the circulating immune complex to the target antigen via a competitive mechanism or a steric inhibition, as well as non-specific blocking of Fc receptor mediated phagocyte function might also contribute to the therapeutic effect.

The pooled IvIg product might contain FcR specific antibodies. Soon after IvIg therapy there is a decrease in immunoglobulin production. The CD4/CD8 ratio is altered and B cell function decreased.

The aim of the present study was to investigate the effect of IvIg treatment on cytokine production.

MATERIALS AND METHODS

Patients

20 pregnant woman were included in this study. The control group included 7 healthy pregnant women. The "patient" group consisted of 13 women at risk for premature pregnancy termination. The latter group included primary habitual aborters as well as women showing clinical symptoms (bleeding or regular uterine contractions) of threatened premature pregnancy termination. Primary habitual aborters were defined as those with a history of 3 or more unexplained spontaneous abortions and no successful pregnancy.

Treatment of pregnancy lymphocytes by immunoglobulin

Lymphocytes were isolated from heparinized venous blood on Ficoll-Paque gradient. The cells were adjusted to a concentration of $1x10^6$/ml in Parker 199 supplemented with 10% FCS (Gibco). The cells were treated with 100 mg/ml of the immunoglobulins for 24 hr at 37C° in a CO_2 incubator, or under the same conditions in the absence of immunoglobulins. After the incubation the cells were washed once in Parker 199 solution and centrifuged on glass microscope slides.

Treatment of non-pregnant lymphocytes by dialysed and heat-treated immunoglobulin

An aliquot of the immunoglobulin preparations was dialysed overnight against phosphate buffered saline at 4°C or incubated at 56°C for 30 min.

Using the previously described conditions, we treated the non-pregnant lymphocytes with dialysed and heat-treated immunoglobulins. After the incubation

the cells were washed once in Parker 199 solution and centrifuged on glass microscope slides.

Immunocytochemistry

Lymphocytes were isolated from heparinized venous blood on Ficoll-Paque gradient. The cells were centrifuged on glass microscope slides. The slides were dried at room temperature, the cells were fixed for 5 min in cold acetone and washed in Tris buffered saline (TBS). All incubations were carried out at room temperature in a humid chamber. After blocking of endogenous peroxidase activity with 1% H_2O_2 the cells were further incubated in TBS containing 1% bovine serum albumin (BSA, Sigma Chemical Co., St. Louis, MO) for blocking nonspecific protein-binding sites.

The primary antibodies were the following; anti IL-10 and anti IL-12 monoclonal antibodies were purchased from R&D Labs (R&D Systems, Abingdon, Oxon, UK). The antibodies were diluted 1:50 respectively in TBS supplemented with 0.5% BSA. The second antibody (HRPO-labelled anti-mouse IgG) was purchased from Dakopatts, Hungary, and applied at dilution of 1:100 respectively for 30 min. The reaction was developed by diaminobenzidine, intensified with silver staining.

The percentage of positive cells was calculated after counting 300 lymphocytes in the microscope at high power magnification.

4-hr single cell cytotoxic assay for NK activity

We used the assay originally described by Grimm and Bonavida.[8] One hundred µl of lymphocytes and the same amount of K562 target cells ($2x10^6$cells/ml each) were centrifuged at 40g for 5 min, and incubated at 37 °C, in 5% CO_2 for 10 min. The pallets were then resuspended and 200 µl of 1% agarose

(Serva) in RPMI 1640 (Gibco) was added to the mixture. One hundred μl of this suspension was spread over microscope glass slides previously coated with 1% agar. Target cells alone were used to detect spontaneous lysis. The gel was allowed to solidify and submerged in RPMI 1640. The slides were incubated for 4 hr at 37°C in 5% CO_2. Then the gels were stained with 0.5% trypan blue for 1 min. After 2 min washes with PBS, the gels were fixed in 2% formaldehyde for 5 min and desalted in distilled water. The slides were read using light microscope with 400x magnification. The proportion of lymphocytes bound to the target cells was expressed as a percentage of total lymphocyte population by counting 300 lymphocytes. Results are expressed as a percentage of target binding cells (TBC). Dead conjugates were scored as a percentage of the total number of conjugates by counting 50 conjugates and results are expressed as a percentage of dead conjugates (cytotoxic TBC%). The percentage of NK cells was calculated according to the formula NK%=TBC% x cytotoxic TBC% / 100. All results for cytotoxic TBC% were corrected for the proportion of target cells that died spontaneously in control plates.

Statistics

The two tailed Student's t-test was used for statistical evaluation of the data. Differences were considered significant if P value was equal or less than 0.05.

RESULTS

To investigate the mechanism of immunoglobulin treatment in RSA, we determined the effect of in vitro immunoglobulin treatment on the rate of IL-10 and IL-12 positive cells among peripheral lymphocytes of healthy pregnant women and women at risk for premature pregnancy termination, including primary habitual aborters as well as women showing clinical symptoms (bleeding or regular uterine contractions) of threatened premature pregnancy termination.

The effect of in vitro immunoglobulin treatment on IL-12 expression in peripheral lymphocytes.

Twenty four h incubation with 5 different immunoglobulin preparations significantly ($p<0.01$) decreased the rate of IL-12 positive cells among lymphocytes of recurrent spontaneous aborters, whereas the treatment had no significant effect on lymphocytes of healthy pregnant women (Fig.1).
The effect exerted by the different batches of immunoglobulin preparations did not significantly differ from each other (Table 1).
Dialysis or heat treatment (56°C, 30 min) of the immunoglobulin preparations did not modify the effect (Table 2).

The effect of in vitro immunoglobulin treatment on IL-10 expression in peripheral lymphocytes.

Twenty four h incubation with 5 different immunoglobulin preparations significantly ($p<0.01$) increased the rate of IL-10 positive cells among lymphocytes of women at risk for threatened premature pregnancy termination, whereas the treatment had no significant effect on normal pregnancy lymphocytes (Fig.2).

There was no significant difference between the effects exerted by the different samples of immunoglobulin preparations (Table 1).

The effect of immunoglobulin treatment on NK activity

In normal human pregnancy, NK activity is significantly lower than in non-pregnant individuals, whereas spontaneous abortion and term labour are associated with increased NK activity.[9]

High NK activity of women at risk for premature pregnancy termination significantly decreased after immunoglobulin treatment in all cases, whereas NK activity of normal pregnancy lymphocytes was not altered (Fig. 3)

DISCUSSION

Cytokines are identified to be of particular importance in interaction between the conceptus and maternal immune system. Cell-mediated immunity is decreased during pregnancy, [10] whereas T-cell-dependent immunoglobulin production remains intact or is increased. [11,12]

Many observations support the concept that pregnancy is associated with an altered Th1/Th2 balance. Pregnancy is associated with a relative increase in Th2-associated immunity, characterised by increased production of the cytokines IL-4 and IL-10.[13] These changes occur concomitantly with increased immunoglobulin production and decreased Th1 action.

Assuming that intravenous immunoglobulin preparations might contain pre-formed anti-cytokine antibodies, and acting on the cytokine balance might be a part of their beneficial effect in RSA, we investigated the effect of in vitro immunoglobulin treatment on cytokine production by pregnancy lymphocytes.

We found a significantly increased expression of IL-10 and a significantly decreased expression of IL-12 on peripheral lymphocytes from women with a history of recurrent abortions or with clinical symptoms of threatened abortion or threatened preterm delivery, whereas lymphocytes from normal healthy pregnant women showed only slightly altered cytokine production.

A significantly increased NK activity has been associated with RSA. It is possible that elevated NK activity is due to increased IL-12 production.

In this study we demonstrated a decreased IL-12 production together with a decreased NK activity in RSA lymphocytes after in vitro immunoglobulin treatment. Wegmann and others have proposed that during normal pregnancy there is a shift of the immune system towards Th2 responses and that the presence of Th1 responses can be a cause for RSA.[5] It has been shown that the presence of Th1 responses induced by IL-1, IL-2, TNF-α and IFN-γ can induce abortion[14] and that a reduction of cell mediated cytotoxic responses, modulating the immune responses

towards a Th2 response with IL-4 and IL-10, prevented a fetal resorption in a murine model of spontaneous abortion.[15] Our finding that immunoglobulin treatment reduces NK activity and thus favours a successful pregnancy is consistent with the observation that cytotoxic activity induced by Th1 responses have deleterious effects on pregnancy and that the prevention of such responses protects pregnancy. Therefore, women at risk for premature pregnancy termination and elevated NK activity may benefit from immunoglobulin treatment because immunoglobulin not only reduces the NK activity as previously reported,[16] but also inhibits the Th1 responses and helps to shift the immune response towards a protective Th2 response.

REFERENCES

1. Sargent, I.L., Wilkins, T., Redman, C.W.G. Maternal immune responses to the foetus in early pregnancy and recurrent miscarriage. Lancet 1988; 11: 1099-1104.

2. Coulam, C.B. Immunological tests in the evaluation of reproductive disorders: A critical review. Am. J. Obstet. Gynecol. 1992; 167: 1844-1851.

3. Aoki, K., Kajiura, S., Matsumoto, Y., Ogasawara, M., Okada, S., Yagami, Y., Gleishner, N. Preconceptional natural-killer activity as a predictor of miscarriage. Lancet 1995; 11: 283-286.

4. Coulam, C.B., Goodman, C., Roussev, R.G., Thomason, E.J., Beaman, K.D. Systemic CD56+ cells can predict pregnancy outcome. Am. J. Reprod. Immunol. 1995; 33: 40-46.

5. Wegmann, T.G., Lin, H., Guilbert, L., Mosmann, T.R. Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a Th2 phenomenon? Immunol. Today 1993; 14: 353-356.

6. Hill, J.A., Polgar, K., Anderson, D.J. T-helper type-1 immunity to trophoblast in women with recurrent spontaneous abortion. JAMA 1995; 273: 1933-1936.

7. Mueller-Eckhardt, G., Heine, O., Neppert, J., Kunzel, W., Mueller-Eckhardt, C. Prevention of recurrent spontaneous abortion by intravenous immunoglobulin. Vox. Sang. 1989; 56: 151-154.

8. Grimm, E., and Bonavida, B., Mechanism of cell-mediated cytotoxicity of the single cell level. I. Estimation of cytotoxic T lymphocyte frequency and relative lytic efficiency. J. Immunol. 1979; 123: 2861-2869.

9. Szekeres-Bartho, J., Varga, P., Pacsa, A.S. Immunological factors contributing to the initiation of labour - lymphocyte reactivity in term labor and threatened pre-term delivery. Am. J. Obst. Gynecol. 1986; 155: 108-113.

10. Luft, B.J. and Remington, J.S. Effect of pregnancy on augmentation of natural killer cell activity be Corynebacterium parvum and Toxoplasma gondii. J. Immunol. 1984;132: 2375-2380.

11. Dresser,, D.E. The potentiating effect of pregnancy on humoral immune responses of mice. J. Reprod. Immunol. 1991;20: 253-266.

12. Paavonen, T., Andersson, L.C., Adlercreutz, H. Sex hormone regulation of in vitro immune response. Estradiol enhances human B cell maturation via inhibition of suppressor T cells in Pokeweed Mitogen-stimulated cultures. J. Exp. Med. 1981;154: 1935-1945.

13. Lin, H., Mosmann, T.R., Guilbert, L., Tuntipopipat, S., Wegmann, T.G. Synthesis of T helper 2-type cytokines at the maternal-fetal interface. J. Immunol. 1993;151: 4562-4567.

14. Chaouat, G., Menu, E., Clark, D.A., Dy, M., Minkowski, M., Wegmann, T.G. Control of fetal survival in CBA x DBA/2 mice by lymphokine therapy. J. Reprod. Fertil. 1990; 89: 447-458.

15. Chaouat, G., Meliani, A.A., Martal, J., Raghupathy, R., Ellioy, J., Mosmann T., Wegmann, T. IL-10 prevents naturally occurring fetal loss in the CBA x DBA/2 mating combination, and local defect in IL-10 production in this abortion prone combination is corrected by in vivo injection of IFN-τ. J. Immunol. 1995; 154: 4261-4268.

16. Ruiz, J.E., Kwak, J.Y.H., Baum, L., Gilman-Sachs, A., Beaman, K.D., Kim, T.B., Beer, A.E. Intravenous immunoglobulin inhibits natural killer cell activity in vivo in women with recurrent spontaneous abortion. Am. J. Reprod. Immunol. 1996; 35: 370-375.

Table 1. The effect of different IgG preparations on cytokine production of pregnancy lymphocytes

		Control	1. IgG	2. IgG	3. IgG	4. IgG	5. IgG
Pathological pregnancy	IL-10	2.52±0.44 n=12	8.16±0.78 n=13	7.56±0.6 n=12	8±0.7 n=12	7.87±0.95 n=12	8.2±0.86 n=12
Pathological pregnancy	IL-12	7.36±0.46 n=13	1.96±0.43 n=13	1.65±0.28 n=13	2.1±0.45 n=13	2.66±0.93 n=13	1.71±0.45 n=11
Normal pregnancy	IL-10	6.27±0.65 n=7	8.01±0.85 n=6	8.37±1.23 n=7	9.08±1.7 n=7	7.91±0.66 n=7	8.62±1.13 n=7
Normal pregnancy	IL-12	5.35±1.43 n=7	4.25±1.02 n=7	4.18±1.06 n=7	4.74±0.9 n=7	3.92±0.84 n=7	4.91±1.86 n=7

Table 2. The effect of dialysis and heat treatment on the effect of IgG preparations

	IL-10	IL-12
Control n=5	2.38±0.54	3.68±0.53
IgG n=5	3.76±1.48	2.2±0.25
Heat treated IgG n=5	2.1±0.2	2.24±0.22
Dyalized IgG n=5	3.06±1.42	2.04±0.29

Fig. 1

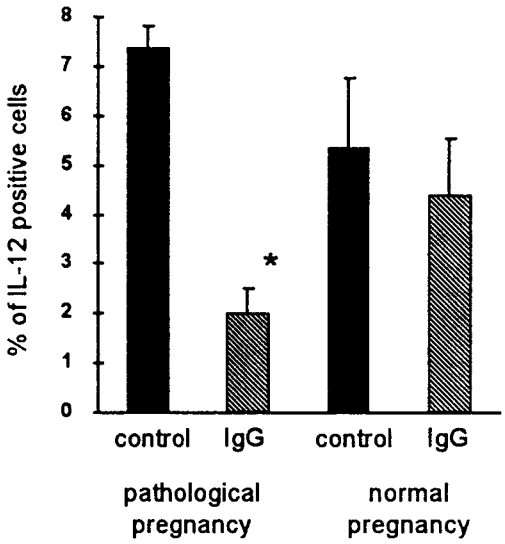


Fig. 1. IL-12 expression on lymphocytes of women with normal and pathological pregnancies after IgG treatment. The bars represents the mean ± SEM of 7 (normal pregnancy) and 13 (pathological pregnancy) determinations. * $p<0.01$

Fig. 2

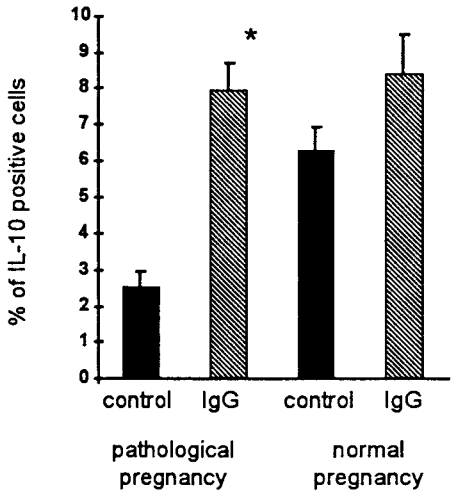


Fig. 2. IL-10 expression on lymphocytes of women with normal and pathological pregnancies after IgG treatment. The bars represents the mean ± SEM of 7 (normal pregnancy) and 13 (pathological pregnancy) determinations. * $p<0.01$

Fig. 3

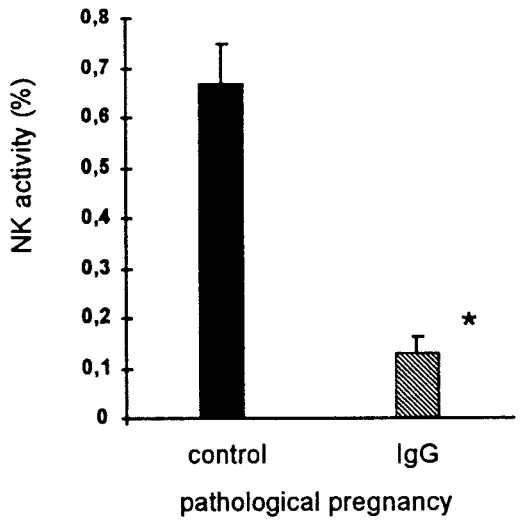


Fig. 3. NK activity of lymphocytes of women with pathological pregnancies after IgG treatment. The bars represents the mean ± SEM of 6 (pathological pregnancy) determinations. * $p<0.01$

VI. METHODS

1. Immunocytochemistry

Lymphocytes were isolated from heparinized venous blood on Ficoll-Paque gradient. The cells were washed once in Parker 199 solution and centrifuged on glass microscope slides. The slides were dried on room temperature, the cells were fixed for 5 min in cold acetone and washed in Tris buffered saline (TBS). All incubations were carried out at room temperature in a humid chamber. After blocking of endogenous peroxidase activity with 1% H_2O_2 the cells were further incubated in TBS containing 1% bovine serum albumin (BSA, Sigma) for blocking nonspecific protein-binding sites.

The primary antibodies were diluted in TBS supplemented with 0.5% BSA. The second antibodies (HRPO-labelled) were purchased from Dakopatts and Sigma, Hungary, and applied at a proper dilution respectively for 30 min. The reaction was developed by diaminobenzidine, intensified with silver staining.

The percentage of positive cells was calculated after counting 300 lymphocytes in the microscope at high power magnification.

2. ELISA

Determination of IL-10 in murine cell supernatants

IL-10 production of murine spleen cells was determined by ELISA, using an Amersham Biotrak system. The assay was performed according to the suggestions of the manufacturer.

3. Production of PIBF

Spleen cells of 10-week-old Balb/c mice were adjusted to a cell count of 1 x 10^6/ml in RPMI supplemented with 10% fetal calf serum (FCS; both from Gibco, Grand Island, NY) and were stimulated by 1 μg/ml of ConA (Sigma) for 48 hr at 37 °C in a CO_2 incubator. The cell count was then adjusted to 10x10^6/ml, and the cells were further incubated with 20 μg/ml of progesterone for 16 hr. At the end of the incubation the supernatants were collected. Progesterone was removed by dialysis. The supernatants were then concentrated 2000 fold on Amicon filters and were used as the source of the murine PIBF.

4. Production of PIBF-specific IgG

Spleen cells of pregnant mice were treated with 20μg/ml of progesterone overnight. The supernatants were collected, concentrated on Amicon filters, and subjected to polyacrilamide gel electrophoresis (PAGE) on 12% polyacrilamide gels. The separated bands were blotted to nitrocellulose filters, the 34 kDa band was cut out, dissolved in DMSO and injected to rabbits weighing 4kg each together with complete Freund adjuvant. Boosters with incomplete Freund adjuvant were given at 2-week intervals. Immunoglobulin G (IgG) was purified on protein A columns.

5. Neutralization of endogenous PIBF activity in pregnant mice

Neutralisation of endogenous PIBF activity was achieved by treating mice on day 8.5 of pregnancy with 0.5 mg of rabbit anti-PIBF IgG. On day 10.5, anti-PIBF treated and control animals were sacrificed, the rate of resorption was recorded and spleens were removed aseptically. Spleen cells were isolated and at a cell count of $1x10^6$/ml activated with 1mg/ml of ConA (Sigma) for 48h. At the end of the incubation supernatants were collected and IL-10 concentrations were determined by ELISA.

6. Treatment of pregnant mice by RU486, anti-PIBF, anti-NK and anti-NC antibodies

Fourteen-week-old Balb/c mice (LATI, Gödöllö, Hungary) were kept under standard conditions (4 animals per cage). Female mice were caged overnight with the males and checked for the presence of vaginal plugs the following morning. The day on which the plug was observed is considered to be Day 0.5 of pregnancy. Pregnancy was later verified by scoring corpora lutea. Various treatments were administered on day 8.5 of pregnancy.

(1) Females were injected intraperitoneally with 3.3 mg/kg of RU486. Another group of mice was injected on day 8.5 of gestation with 0.5 mg of rabbit anti-PIBF IgG. Mice treated with the same amount of normal rabbit serum or untreated mice of similar gestational age were used as controls.

(2) A group of anti-PIBF-treated mice was at the same time injected with monoclonal antibodies to cells mediating natural killer (NK), natural cytotoxic (NC), or natural T cell (NT) activity. The monoclonal antibodies were:

(a) PK136 (anti-NK-1.1) recognizes 76- to 80- kDa type II integral membrane C-type lectin protein encoded by a member of the mouse NKR-PI gene family.[86,87]

(b) 1C4 (anti-NC-1.1) which recognizes a 45-kDa surface receptor and blocks splenic NC activity approximately 70% both in vitro and in vivo.[88,89]

(c) 2B6-F2 (anti-ly-6c) identifies a subpopulation of murine Ly-6c$^+$ NK1.1$^+$ natural (NT) cells. In the presence of complement, 2B6-F2 reduces splenic NK activity by approximately 50% in Balb/c mice.[90,91]

7. 4-hr single cell cytotoxic assay for NK activity

We used the assay originally described by Grimm and Bonavida.[92] One hundred μliters of lymphocytes and the same amount of K562 target cells ($2x10^6$/ml each) were centrifuged at 40g for 5 min, and incubated at 37 °C, in 5% CO_2 for 10 min. The pellets were then resuspended and 100 μl of 1% agarose (Serva) in RPMI 1640 (Gibco) was added to the mixture. One hundred μliters of this suspension was spread over microscope glass slides previously coated with 1% agar. Target cells alone were used to detect spontaneous lysis. The gel was allowed to solidify and was submerged in RPMI 1640. The slides were incubated for 4 hr at 37 °C in 5% CO_2. Then the gels were stained with 0.5% trypan blue for 1 min. After 2 min washes with PBS, the gels were fixed in 2% formaldehyde for 5 min and desalted in destilled water. The slides were read using light microscope with 400x magnification. The proportion of lymphocytes bound to the target cells was expressed as a percentage of total lymphocyte population by counting 300 lymphocytes. Results are expressed as a percentage of target binding cells (TBC). Dead conjugates were scored as a percentage of the total number of conjugates by counting 50 conjugates and results are expressed as a percentage of dead conjugates (cytotoxic TBC%). The percentage of

NK cells was calculated according to the formula NK%=TBC% x cytotoxic TBC% / 100. All results for cytotoxic TBC% were corrected for the proportion of target cells that died spontaneously in control plates.

8. TNF-α

The concentration of TNF-α in human serum was determined by measuring cytotoxicity to L929 cells.

9. Statistics

The two tailed Student's t-test was used for statistical evaluation of our data, with $p<0.01$ considered as statistically significant.

VII. REFERENCES

1. Medawar, B.P., Some immunological and endocrinological problems raised by the evolution of viviparity in vertebrates. Evolution 1953; 7: 320-338.

2. Chaouat, G., Clark, D.A. and Wegmann, T.G. Genetic aspects of the CBA/Jx DBA/2 and b10xB10.A. Models of murine pregnancy failure and its prevention by lymphocyte immunization, in Early Pregnancy Loss, Mechanisms and Treatment. Beard, R.W. and Sharp, F. Eds. Peacock Press, Ashton-under-Lyne, U.K. 1988; 89.

3. Chaouat, G., Kolb, J.P., Lankar, D., Canepa, S., Menu, E., Kaplan, L., Andreux, G., Denvers, L. and Chaffraux, S. Mode d'action caracterisation biochimique, prepurification et progres vers biologie moleculaire des immunregulateurs placentaires murins. Premieres etudes en systeme human. Coll. I.N.S.E.R.M. 1987; 154: 203.

4. Clark, D.A., Slapsys, R.M., Croy, B.A., Koeck, J. and Rossant, J. Local active suppression by suppressor cells in the decidua: a review. Am. J. Reprod. Immunol. 1984; 5: 78-83.

5. Redman, C.W.G., Areans, J. and Sargent, I.L. Maternal anti-fetal immune responses during human pregnancy. Coll. I.N.S.E.R.M. 1987; 154: 25.

6. Stites, D.P., Pavia, C.S., Clements, L.E., Kuhn, R.W. and Siiteri, P.K. Immunologic regulation in pregnancy. Arthhr. Rheum. 1979; 22: 1300-1307.

7. Csapo, A.I. and Wiest, W.G. An examination of the quantitative relationship between progesterone and the maintenance of pregnancy. Endocrinology. 1969; 85: 735-746.

8. Stites, D.P. and Siiteri, P.K. Steroids as immunosuppressants in pregnancy. Immunol. Rev. 1983; 75: 117-138.

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

10. Chumbley, G., King, A., Robertson, K., Holmes, N. and Loke, Y.W. Resistance of HLA-G and HLA-A2 transfectants to lysis by decidual NK cells. Cell. Immunol. 1994; 155: 312-322.

11. Timonen, T., Saksela. E., Ranki, A., Hayry, P. Fractionation, morphological and functional characterization of effector cells responsible for human killer activity against cell-line targets. Cell Immunol 1979; 48: 133-148.

12. Trinchieri G. Biology of Natural killer cells. Adv. Immunol. 1989; 47: 187-376.

13. Szekeres-Bartho, J., Kinsky, R., and Chaouat, G. The effect of a progesterone-induced immunologic blocking factor on NK-mediated resorption. Am. J. Reprod. Immunol. 1990; 24: 105-107.

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

15. Mosmann, T.R., Cherwinski, H., Bond, M.W., Giedlin, M.A. and Coffman, R.L. Two types of murine helper T cell clone. J.Immunol. 1986; 136: 2348-2357.

16. Del Prete, G.F., M. De Carli, F. Almerigogna, C.K. Daniel, M.M. D'Elios, G. Zancuoghi, F. Vinante, G. Pizzolo, and S. Romagnani. Preferential expression of

CD30 by human CD4+ T cells producing Th2-type cytokines. FASEB J. 1995; 9: 81-88.

17. Da Silva, J.A. and Spector, T.D. The role of pregnancy in the course and aetiology of rheumatoid arthritis. Clin. Rheumatol. 1992; 11: 189-194.

18. Varner, M.W. Autoimmune disorders and pregnancy. Semin. Perinatol. 1991; 15: 238-250.

19. Minkoff, H., Nanda, D., Menez, R. and Fikrig, S. Pregnancies resulting in infants with acquired immunodeficiency syndrome or AIDS-related complex: follow-up of mothers, children, and subsequently born siblings. Obstet. Gynecol. 1987; 69: 288-291

20. Weber, D.J., Wolfson, J.S., Swartz, M.N. and Hooper, D.C. Pasteurella multocida infections. Report of 34 cases and review of the literature. Medicine Baltimore 1984; 63: 133-154.

21. Watkinson, M. and Rushton, D.J. Plasmodial pigmentation of placenta and outcome of pregnancy in West African mothers. Br. Med. J. Clin. Res. 1983; 287: 251-254.

22. Luft, B.J. and Remington, J.S. Effect of pregnancy on resistance of Listeria monocytogenes and Toxoplasma gondii infections in mice. Infect. Immunol. 1982; 38: 1164-1171.

23. Lin, H., Mossman, T.R., Guilbert, L., Tuntipopipat, S., Wegmann, T.G. Synthesis of T helper-2 cytokines at the maternal fetal interface. J. Immunol. 1993; 151: 4562-4573.

24. Lin, H., Mossmann, T.R., Guilbert, L., Tuntipopipat, S., Wegmann, T.G. Synthesis of T helper 2-type cytokine at the maternal-fetal interface. J. Immunol. 1993; 151: 4562-4573.

25. Delassus, S., Countinho, G.S., Saucier, S., Darche, S., Kourilky, P. Differential cytokine expression in maternal blood and placenta during murine gestation. J. Immunol. 1994; 152: 2411-2420.

26. Fiorentino, D.J., Boond, M.W. and Mosmann, T.R. Two types of mouse Th cells. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. J. Exp. Med. 1989; 170: 2081-2095.

27. Chaouat, G., Menu, E., Dy, M., Minkowski, M., Clark, D.A. Wegmann, T. Control of fetal survival in CBA x DBA/2 mice by lymphokine therapy. J. Fertil. Steril. 1990; 89: 447-458.

28. Parand, M., Chedid, L. Protective effects of chlorpromazine against endotoxin induced abortion. Proc. Soc. Exp. Med. 1964; 116: 906-915.

29. Tangri, S., Raghupathy, R. Expression of cytokines in placenta of mice undergoing immunologically mediated fetal resorption. Biol. Reprod. 1993; 49: 840-850

30. Haimovici, F., Hill, J.A. and Anderson, D.J. The effect of soluble products of activated lymphocytes and macrophages on blastocyst implantation events in vitro. Biol. Reprod. 1991; 44: 69-75.

31. Yui, J., Garcia-Llooret, M., Wegmann, T.G. and Guilbert, L.J. Cytotoxicity of tumor necrosis factor-α (TNF-α) and gamma-intrferon (IFN-γ) against primary human placental trophoblasts. Placenta 1994; 15: 819-826.

32. Raghupathy, R. and Tangri, S. Immunodystrophism, T cells, cytokines and pregnancy failure. Am. J. Reprod. Immunol. 1996; 35: 291-296.

33. Wegmann, T.G., Lin, H., Guilbert, L., Mossman, T.H. Bi-directional cytokine interactions in the maternal-fetal relationship: successful allopregnancy is a Th2 phenomenon. Immunol. Today 1993; 14: 353-355.

34. Moriyama, I., Sugawa, T. Progesterone facilitates implantation of xenogenic cultured cells in hamster uterus. Nature New. Biol. 1972; 236: 150-153.

35. Hansen, P.J., Bazer, F.W., Segerson, E.C. Skin graft survival in the uterine lumen of ewes treated with progesterone. Am. J. Reprod. Immunol. 1986; 12: 48-54.

36. Siiteri, P.K., Febre, F., Clemens, L.E., Chang, R.J., Gondos, B., Stites, D.P. Progesterone and the maintaince of pregnancy: Is progesterone nature's immunsuppressant? Ann. N.Y. Acad. Sci. 1977; 286: 384-397.

37. Stites, D.P., Bugbee, S., Siiteri, P.K. Differential actions of progesterone and cortisol on lymphocyte and monocyte interaction during lymphocyte activation - relevance to immunsuppression in pregnancy. J. Reprod. Immunol. Microbiol. 1985; 5: 15-18.

38. Neifeld, J.P. and Toromey, D.C. Effects of steroide hormones on phytohaemagglutinin stimulated human peripherial blood lymphocytes. Transplantation 1979; 27: 309-314.

39. Schiff, R.I., Mercier, D. and Buckley, R.H. Inability of gestational hormones to account for the inhibitory effects of pregnancy plasmas on lymphocyte responses in vitro. Cell. Immunol. 1975; 20: 69-80.

40. Szekeres, J., Csernus, V., Pejtsik, B., Emody, L. and Pacsa, A.S. J. Reprod. Immunol. 1981; 3: 333-339.

41. Szekeres-Bartho, J., Csernus, V., Hadnagy, J. and Pacsa, A.S. Immunosuppressive effect of serum progesterone during pregnancy depends on the progesterone binding capacity of the lymphocytes. J. Reprod. immunol. 1983; 5: 81-88.

42. Szekeres-Bartho, J., Szekeres, Gy., Debre, P., Autran, B. and Chaouat, G. Reactivity of lymphocytes to a progesterone receptor-specific monoclonal antibody. Cell. Immunol. 1990; 125: 273-283.

43. Szekeres-Bartho, J., Reznikoff-Etievant, M.F., Varga, P., Pichon, M.F., Varga, T. and Chaouat, G. Lymphocytic progesterone receptors in normal and pathological human pregnancy. J. Reprod. immunol. 1989; 16: 239-247.

44. Szekeres-Bartho, J., Kilar, F., Falkay, G., Csernus, V., Torok, A., Pacsa, A.S. The mechanism of the inhibitory effect of progesterone on lymphocyte cytotoxicity: I. Progesterone-treated lymphocytes release a substance inhibiting cytotoxicity and prostaglandin synthesis. Am. J. Reprod. Immunol.1985; 9: 15-18.

45. Szekeres-Bartho, J., Chaouat, G. Lymphocyte-derived progesterone-induced blocking factor corrects resorption in a murine abortion system. Am. J. Reprod. Immunol. 1990; 23: 26-28.

46. Szekeres-Bartho, J., Kinsky, R., Chaouat, G. The effect of a progesterone-induced immunologic blocking factor on NK-mediated resorption. Am. J. Reprod. Immunol. 1990; 24: 105-107.

47. Szekeres-Bartho, J., Faust, Zs., Varga, P. The expression of a progesterone-induced immunomodulatory protein in pregnancy lymphocytes. Am. J. Reprod. Immunol. 1995; 34: 342-348.

48. Szekeres-Bartho, J., Wegmann, T.G. A progesterone-dependent immunomodulatory protein alters the Th1/Th2 balance. J. Reprod. Immunol. 1996; 31: 81-95.

49. Gendron, R. and Baines, M. Infiltrating decidual natural killer cells are associated with spontaneous abortion in mice. Cell. Immunol. 1988; 113: 261-267.

50. De Fuogerolles, R. and Baines, M. J. Modulation of the natural killer cell activity in pregnant mice alters the spontaneous abortion rate. Reprod. Immunol. 1987; 11: 147-153.

51. Kinsky, R., Delage, G., Rosin, N., Thang, M.N., Hoffmann, M., and Chaouat, G., A murine model of NK cell mediated resorption. Am. J. Reprod. Immunol. 1990; 23: 73-77.

52. Szekeres-Bartho, J., Kinsky, R., and Chaouat, G., The effect of a progesterone-induced immunologic blocking factor on NK-mediated resorption. Am. J. Reprod. Immunol. 1990; 24: 105-107.

53. Tonegawa, S., Berns, A., Bonneville, M., Farr, A.G., Ishida, I., Ho, K., Itohara, S., Janeway, C.A., Kanagawa, O. and Kubo, R. Diversity, development, ligands and probable functions of γδ T cells. Adr. Exp. Med. Biol. 1991; 292: 53-61.

54. Allison, J.P. and Havran, W.L. The immunobiology of T cells with invariant γδ antigen receptor. Annu. Rev. Immunology 1991; 9: 679-705.

55. Haas, W., Pereira, P. and Tonegawa, S. γδ cell. Annu. Rev. Immunology 1993; 11: 637-685.

56. Kent, D.H., Cranfill, R.L., Carding, S.R., Born, W.K., O'Brien, R.L. Characterization of γδ T lymphocytes at the maternal-fetal interface. J. Immunol. 1992; 149: 2872-2878.

57. Mincheva-Nilsson, L., Hammerström, S. and Hammerström, M-L. Human decidual leukocytes from early pregnancy contain high numbers of γδ cells and show selective down-regulation of alloreactivity. J. Immunol. 1992; 149: 2203-2211.

58. Itohara, S., Farr, A.G., Laffaille, J.J., Boneville, M., Takagaki, Y., Haas, W. and Tonegawa, S. Homing of a γδ thymocyte subset with homogenous T-cell receptors to mucosal epithelia. Science 1990; 343: 754-757.

59. Schild, H., Mavaddat, N., Litzenberg, C., Ehrich, E.W., Davis, M.M., Bluestone, J.A., Matis, L., Draper, K.R. and Chien, Y.H. The nature of major histocompatibily complex recognition by γδ T cells. Cell. 1994; 76: 29-37.

60. Heyborne, K., Fu, Y-X., Nelson, A., Farr, A., O'Brien, R. and Born, W. Recognition of trophoblast by γδ T cells. J. Immunol. 1994; 153: 2918-2926.

61. Sheppard, B.L. and Bonnar J. An ultrastructural study of uteroplacental spiral arteries in hypertensive and normotensive pregnancy and fetal growth retardation. Br. J. Obstet. Gynecol. 1981;88: 695-705.

62. Hertz-Picciotto, I. and Samuels, S.J. Incidence of early loss in pregnancy. N. Eng. J. Med. 1988; 319: 1483-1484.

63. Kilpatrick, D.C and Liston, W.A. Charasteristics of Scottish patients presenting with reccurent miscarriage. J. Obstet. Gynaecol. 1994; 14: 221-225.

64. Kilpatrick, D.C. and Liston, W.A. Parental HLA sharing, feto-maternal compatibility and neonatal birthweight in families with a history of recurrent spontaneous abortion. Dis. Markers. 1993; 11: 125-130.

65. Coulam, C., Clark, D.A., Scott, J.S., Schesselmann, J., the Reccurent Miscarriage Trialists Group. Worldwide collaborative study and metaanalysis on allogenic leukocyte immunothearpy for reccurrent spontaneous abortion. Am. J. Reprod. Immunol. 1994; 32: 55-72.

66. Szekeres-Bartho, J., Varga, P. and Pacsa, A.S. immunological factors contributing to the initiation of labour-lymphocyte reactivity in term labour and threatened pre-term delivery. Am. J. Obstet. Gynecol. 1986; 155: 108-113.

67. Szekeres-Bartho, J., Autran, B., Debre, P., Andreu, G., Denver, L. and Chaouat, G. Immunmodulatory effects of a suppressor factor from healthy pregnant woomen's lymphocytes afetr progesterone induction. Cell Immunol. 1989; 122: 281-294.

68. Kinsky, R., Delage, G., Rosin, N., Thang, M.N., Hoffmann, M. and Chaouat, G. A murine model for NK-mediated resorption. Am. J. Reprod. Immunol. 1990; 23: 73-77.

69. Szekeres-Bartho, J., Varga, P. and Pacsa, A.S. Immunological factors contributing to the initiation of labour and threatened pre-term delivery. Am. J. Obstet. Gynecol. 1986; 155: 108-113.

70. Gendron, R. and Baines, M. Infiltrating decidual natural killer cells are associated with spontaneous abortions in mice. Cell. Immunol. 1988; 113: 264-268.

71. De Fougerolles, R. and Baines, M. Modulation of natural killer activity influences resorption rates in CBA x DBA/2 matings. J. Reprod. Immunol. 1987; 11: 147-153.

72. Szekeres-Bartho, J., Autran, B., Debre, P., Andreu, G., Denver, L. and Chaouat, G. Immunoregulatory effects of a suppressor factor from healthy pregnant women's lymphocytes after progesterone induction. Cell. Immunol. 1989; 122: 281-294.

73. Barth, R.J., Coppola, M.A., Green, W.R. In vivo effects of locally secreted IL-10 on the murine antitumor immune response. Ann. Surg. Oncol. 1996; 3: 381-386

74. Fitch, F.W., McKisic, M.D., Lancki, D.W. Gajewski,-T.F. Differential regulation of murine T lymphocyte subsets. Annu. Rev. Immunol. 1993; 11: 29-48

75. Silen, M.L., Firpo, A., Morgello, S., Lowry, S.F. and Francus, T. Interleukin 1α and tumor necrosis factor α cause placental injury in the rat. Am. J. Pathol. 1989; 135: 239-244.

76. Parant, M. Possible mediators in endotoxin induced abortion. Res. Immunol. 1990; 141: 164-168.

77. Romero, R., Mazor, M., Wu, Y.K., Avila, C., Oyarzun, E. and Mitchell, M.D. Bacterial endotoxin and tumor necrosis factor stimulate prostaglandin production by decidua. Prostaglandins Leukot. Essent. Fatty Acids 1989; 37: 183-186.

78. Coulam, C.B. and Coulam, C.H. Update on immunotherapy for recurrent pregnancy loss. Am. J. Reprod. Immunol. 1992; 27: 124-127.

79. Heilmann, L., Kriechbaum, A., Hojnacki, B., Bode, V. and Wolf, H. Clinical experiences with passive immunotherapy in habitual abortion. Z. Geburts. Perinatol. 1993; 197: 55-58.

80. Carp, H.J.A., Ahiron, R., Mashiach, S., Schonfeld, Y., Gazit, E. and Toder, V. Intravenous immunoglobulin in women with five or more abortions. Am. J. Reprod. Immunol. 1996; 35: 360-362.

81. Coulham, C.B. Immunotherapy for recurrent spontaneous abortion. Early Pregnancy. Biol. Med. 1995; 1: 13-26.

82. German RSA/IVIG group. Intravenous immunoglobulin in the prevention of recurrent miscarriage. Br. J. Obstet. Gynaecol. 1994; 101: 1072-1077.

83. Dwyer, J.M. Manipulating the immune system with immune globulin. N. Eng. J. Med. 1992; 326: 107-116.

84. Anderson, J.P. and Anderson, U.G. Human intravenous immunoglobulin modulates monokine production in vitro. Immunology 1990; 71: 372-376.

85. Ruiz, J.E., Kwak, J.Y.H., Baum, L., Gilman-Sachs, A., Beaman, K.D., Kim, T.B., Beer, A.E. Intravenous immunoglobulin inhibits natural killer cell activity in vivo in women with recurrent spontaneous abortion. Am. J. Reprod. Immunol. 1996; 35: 370-375.

86. Koo, G.C., and Peppard, J.R., Establishment of monoclonal anti-Nk-1.1 antibody. Hybridoma 1984;3:301-303

87. Ryan, J. C., Turck, N., Niemi, E.C., Yokoyama, W.M., and Seaman. W.E., Molecular cloning of the NK1.1 antigen, a member of the NKR-P1 family of natural killer cell activation molecules. J. Immunol. 1992; 149: 1631-1635.

88. Smart, Y.C., Stevenson, K.L., Farrelly, M.L., Brien, J.H., and Burton, R.C., Production of a monoclonal allo-antibody to murine natural cytotoxic cells. Immunol. Cell. Biol. 1990; 68: 277-284.

89. Holmgreen, S.P., Wang, X., Clarke, G.R., Nolthorp, R.S., Roberts, T.K., Burton, R.C., Robinson,. J.P., and Smart, Y.C., Phosphorylation of the NC-1.1 receptor and regulation of natural cytotoxicity by protein kinase C and cyclic GMP-dependent protein kinase. J. Immunol.1997;158: 2035-2041.

90. Martiniello, R., Smart, Y.C., and Burton, R.C., Monoclonal antibody 2B6-F2 identifies a subset of murine natural killer cells with receptors for YAC-1 and WEHI-164 targets. Cell. Immunol. 1994; 156: 155-169.

91. Martinello, R., Burton, R.C., and Smart, Y.C., Ly-6C+ natural T (NT) cells mediate immune surveillance against NK-sensitive and NK-resistant transplantable tumours in certain strains of mice. Int. J. Cancer.1996; 66: 532-537.

92. Grimm, E., and Bonavida, B., Mechanism of cell-mediated cytotoxocity of the single cell level. I. Estimation of cytotoxic T lymphocyte frequency and relative lytic efficiency. J. Immunol. 1979; 123: 2861-2869.

PUBLICATIONS:

1. Balikó, Z., Szereday, L., Szekeres, J. T gamma/delta lympocyták aránya aktív Mikobactérium tuberkulózisban szenvedô betegek periferiás vérében. Medicina thoracalis 1995; 49: 216.

2. Szekeres-Bartho, J., Faust, Zs., Varga, P., Szereday, L., Kelemen, K. The immunological pregnancy protective effect of progesterone is manifested via controlling cytokine production. Am. J. Reprod. Immunol. 1996; 35:3 48-351.

3. Baliko, Z., Szereday, L., Szekeres-Bartho, J. γ/δ TCR positive cells in peripheral blood of patients with active Mycobacterium tuberculosis infection. Thorax 1997; 52: 375-377.

4. Julia Szekeres-Bartho, G. Par, L. Szereday, C.Y. Smart, I. Achatz. Progesterone and non-specific immunological mechanisms in pregnancy. Am. J. Reprod. Immunol. 1997; 38: 176-182.

5. Szereday, L., Varga, P., Szekeres-Bartho, J. Cytokine production by lymphocytes in pregnancy. Am. J. Reprod. Immunol. 1997; 38: 418-422.

6. Baliko, Z., Szereday, L., Szekeres-Bartho, J. Cytokine production by peripheral blood lymphocytes of patients with active Mycobacterium tuberculosis infection. (in press)

7. Szereday, L., Späth, P., Szekeres-Bartho, J. NK activity and cytokine production of pregnancy lymphocytes after in vitro immunoglobulin treatment. (submitted)